Febrile Illnesses at the Colombo North Teaching Hospital in Sri Lanka

(The Ragama Fever Study)

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Abstract

Acute undifferentiated febrile illnesses in the tropics and sub-tropics are caused by a wide range of infectious diseases that often have indistinguishable clinical features. In developing countries there may also be insufficient microbiology facilities to identify these infections leading to missed diagnoses, inefficient use of healthcare resources, over-use of empirical treatments, a lack of information on antimicrobial resistance and inaccurate epidemiological data for guiding prevention strategies. These problems occur in Sri Lanka, but a prospective, systematic, representative and comprehensive study of febrile illnesses has never been performed.

The Ragama Fever Study was performed at a major hospital in western Sri Lanka that served both urban and rural areas. Its aims were to identify the causes of febrile illnesses in a large sample of patients admitted to the hospital over a 1-year period, develop clinical prediction rules that could distinguish between the most common infectious diseases and assist in the evaluation of rapid (point-of-care) diagnostic tests that were appropriate to this setting.

617 (86.7%) of 711 febrile patients admitted to a quarter of the hospital medical wards were recruited. 56.4% had confirmed infections with organisms identified including dengue (22.2%), chikungunya (16.7%), leptospirosis (5.2%), various bacteraemias (4.2%), Q fever (2.9%), rickettsial infections (2.3%), tuberculosis (1.1%) and urinary tract infections (0.8%). 7.6% had confirmed infections with no organisms identified including cellulitis (2.4%), respiratory tract infections with radiographic changes (2.1%) and pulmonary tuberculosis with radiographic changes (1.6%). 4.1% had confirmed non-infectious diseases and 37.2% had unconfirmed diseases including “viral fever” (13.3%), undifferentiated fever (7.8%), respiratory tract infections (6.8%), urinary tract infections (3.4%), leptospirosis (2.8%) and gastroenteritis (1.0%).

Clinical prediction rules for identifying dengue fever and chikungunya were developed using imputation, multiple logistic regression, scoring algorithms and receiver operating characteristic (ROC) curve analysis. The dengue fever rule had sensitivity = 49.6%, specificity = 93.9%, positive predictive value (PPV) = 70.8% and negative predictive value (NPV) = 86.1%. The chikungunya rule had sensitivity = 35.0%, specificity = 95.0%, PPV = 60.0% and NPV = 87.1%. ROC curve analysis could not identify any probability cut-offs that would produce clinical prediction rules with acceptable combinations of both sensitivity and specificity.

A commercial (Panbio) rapid serology test for dengue fever showed sensitivity = 43.4%, specificity = 88.8%, PPV = 54.6% and NPV = 83.5% on samples from admission and significantly better diagnostic performance on follow-up. When repeated in conjunction with a PanBio rapid NS1 antigen detection test, the diagnostic performance improved with sensitivity = 89.9%, specificity = 75.0%, PPV = 69.0% and NPV = 92.3% on admission.

This study confirmed the wide range of infections that present as febrile illnesses in Sri Lanka and showed the limitations of clinical prediction rules and rapid diagnostic tests in identifying these on admission. I hope that it will prove a foundation for further work on these important topics.

Preface

This thesis has been produced in accordance with the University of Liverpool ordinances and regulations for the degree of Doctor of Medicine (MD). This work has not previously been accepted for any degree nor is it currently being submitted elsewhere for any degree. This thesis is the result of my own work except where otherwise stated.

I designed this study, raised the necessary funding and arranged the research ethics clearance. In Sri Lanka I led the recruitment of patients and collection of clinical data and laboratory samples on a daily basis for the 1-year period of the study and also prepared samples for storage and shipment. Diagnostic testing of these samples was performed in carefully selected local and international reference laboratories to provide confirmed diagnoses with the greatest possible accuracy. I visited each of these laboratories on more than one occasion to observe and (where possible) assist with the diagnostic tests being performed. I also performed a rapid diagnostic dengue serology test on all serum samples obtained and additional rapid diagnostic tests were performed retrospectively at the Mahidol-Oxford Research Unit. I designed an electronic database and entered all of the clinical and laboratory data with 5% data entry checks by my wife. I undertook basic epidemiological, geographical and statistical analyses myself and then performed multiple logistic regression and developed clinical prediction rules with support from a medical statistician. I wrote this thesis with regular reviews provided by my supervisors.

I give my consent for this thesis to be available for reproduction and inter-library loan and for the title and abstract to be made available to outside organisations. I will also be depositing an e-thesis version of this work with the University of Liverpool.

I greatly regret that my role as the only British military infectious diseases physician during major campaigns in Iraq and Afghanistan and other deployments across four continents has meant that I could not complete the write-up of this thesis any earlier.

Acknowledgements

This was an ambitious and wide-ranging study and so I was assisted by a large number of people specialising in the 12 different infectious diseases covered here.

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Finally, I am very grateful for the support shown by my family and work colleagues at Birmingham Heartlands Hospital, Warwick Medical School and the Defence Medical Services during the writing-up of this thesis.

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List of Abbreviations

ALT	Alanine transaminase
ANDV	Andes hantavirus
AST	Aspartate transaminase
AUFI	Acute undifferentiated febrile illness
CAAT	Cross-agglutinin absorption test
CHIKV	Chikungunya virus
CI	Confidence interval
CMV	Cytomegalovirus
CPK	Creatine phosphokinase
CRP	C-reactive protein
DEC	Diethylcarbamazine
DENV	Dengue virus
DOBV	Dobrava-Belgrade hantavirus
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immuno-sorbent assay
ESBL	Extended spectrum beta-lactamase
FUO	Fever of unknown origin
GN	Grama Niladari (postal code)
HAI	Haemagglutination inhibition
HANV	Hantavirus (generic)
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HFRS	Haemorrhagic fever with renal syndrome
HTNV	Hantaan hantavirus
HPS	Hantavirus pulmonary syndrome
ICT	Immuno-chromatic test
IFA	Immuno-fluorescence assay
Ig	Immunoglobulin
IMS	Indian Medical Service
IGRA	Interferon-gamma release assay
ISO	International Organization for Standardization

ITU	Intensive therapy unit
IV	Intravenous
JEV	Japanese encephalitis virus
L	Litre
LRTI	Lower respiratory tract infection
MAT	Micro-agglutination test
MeSH	Medical Subject Headings
MLST	Multi-locus sequence typing
NDM-1	New Delhi metallo-β-lactamase-1
NLM	United States National Library of Medicine
NPV	Negative predictive value
PCR	Polymerase chain reaction
PO	*per orum*
PPV	Positive predictive value
PRNT	Plaque reduction neutralisation technique
PUO	pyrexia of unknown origin
PUUV	Puumala hantavirus
RAMC	Royal Army Medical Corps
ROC	Receiver operator characteristic
RT	Reverse transcriptase
SEOV	Seoul hantavirus
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SNV	Sin Nombre hantavirus
TB	Tuberculosis
THAIV	Thailand hantavirus
TPMV	Thottapalayam hantavirus
TST	Tuberculin skin testing
TULV	Tula hantavirus
U	Units
UFI	Undifferentiated febrile illness
UTI	Urinary tract infection
WCC	White cell count
WHO	World Health Organisation

Chapter 1

Introduction & Literature Review

1.1 Aims and Definitions

1.1.1 Aims

The Ragama Fever Study was conducted from 2006-7 at the Colombo North Teaching Hospital in Ragama, which is located within the Gampaha District of western Sri Lanka. It had three major aims :

1. To identify the causes of febrile illnesses in a representative sample of patients admitted to the hospital over a 1-year period

2. To develop clinical prediction rules that could diagnose the most common infectious diseases found

3. To assist in the evaluation of rapid (point-of-care) diagnostic tests that were appropriate to this setting

1.1.2 Fever

In clinical practice there can be an over-simplistic view that normal body temperature is 37.0 °C with no variation between individuals, site of measurement or over time. However, normal body temperature is known to vary between individuals and sites with ranges of 33.2 to 38.2 °C for oral temperatures, 34.4 to 37.8 °C for rectal temperatures, 35.4 to 37.8 °C for tympanic temperatures and 35.5 to 37.0 °C for axillary temperatures [Sund-Levander *et al.*, 2002]. Normal body temperature also varies within an individual by >1.0 °C due to activity, diurnal rhythm and the menstrual cycle [Refinetti & Menaker, 1992].

In view of this physiological variation, it is not surprising that different definitions of fever have been used in previous fever studies in the tropics (Table 1.1.2). Although definitions vary from >37.5 °C (oral) to >38.3 °C (axillary), it seems unlikely that such raised temperatures in symptomatic patients attending hospital would be due to physiological variation. However, these different definitions of fever could lead to variable recruitment of patients with diseases associated with “low-grade” fevers of

37.5–38.0 °C (oral). Therefore standardising the definition of fever would be useful to ensure that different fever studies are comparable.

Table 1.1.2. Definitions of fever used in previous studies in the tropics

Definition of Fever	Reference
>37.5 °C (oral)	Watt & Jongsakul, 2003
>38.3 °C (oral)	Leelarasamee *et al.*, 2004
≥38 °C (oral)	Murdoch *et al.*, 2004
>38 °C (axillary)	Wagenaar *et al.*, 2004
≥38 °C (unspecified)	Ellis *et al.*, 2006
>38.0 °C (axillary)	Nga *et al.*, 2006
unspecified	Suttinont *et al.*, 2006
>38 °C (axillary)	Blacksell *et al.*, 2007
unspecified	Joshi *et al.*, 2008
≥38 °C (oral)	Manock *et al.*, 2009
>38.3 °C (unspecified)	Chrispal *et al.*, 2010
≥38 °C (unspecified)	Kasper *et al.*, 2010

The measurement of body temperature in tropical countries often reflects the limited healthcare resources available. For example, there may only be a single thermometer per ward and patients may then be unwilling to have oral temperatures taken even if adequate sterilisation procedures are in place. Likewise no fever study in the tropics has yet been performed using tympanic temperature measurements, which are now the standard in developed countries. These issues need to be considered when defining fever for the purposes of a fever study in a developing country.

1.1.3 Undifferentiated Febrile Illnesses

A febrile illness is simply defined as any illness with an associated fever (*eg.* >38.0 °C oral temperature). Some febrile illnesses have a specific organ focus, such as meningitis, pneumonia, gastroenteritis, genito-urinary infections and skin & soft tissue infections, which will normally lead to an early clinical diagnosis before

microbiological confirmation. In contrast, undifferentiated febrile illnesses (UFIs) are usually defined as febrile illnesses with no obvious focus of infection after initial clinical assessment (*ie.* a history & physical examination) and possibly basic imaging tests (*eg.* a chest radiograph).

It is important to distinguish the definition of a UFI from that of a fever (or pyrexia) of unexplained (or unknown) origin (FUO or PUO) since the latter was originally defined as an illness of >3 weeks duration, with fever >38.3 °C on several occasions and no diagnosis after 1 week of study in hospital [Petersdorf & Beeson, 1961]. The term UFI is used in the initial stages of patient management and the term "acute UFI" is sometimes used to indicate that the fever has been present for <2 weeks. UFIs are usually caused by infections that do not have a specific organ focus, whereas FUOs include a higher proportion of non-infectious causes of fever, such as malignant and inflammatory disorders [Petersdorf & Beeson, 1961]. A UFI may persist and defy diagnosis long enough to become a FUO, but the terms should remain distinct.

Unfortunately, the United States National Library of Medicine (NLM) Medical Subject Headings (MeSH) system does not include a term to cover UFIs as defined above. The closest term available is "Fever of Unknown Origin", which it defines as "fever in which the etiology cannot be ascertained", but despite this more general definition the term FUO is still most closely associated with the original Petersdorf & Beeson definition, which requires a fever for >3 weeks and no diagnosis after 1 week of hospital assessment. Some authors have used the term "acute FUO" to remove these time limits, but the potential for confusion remains and so the term (acute) undifferentiated febrile illness is preferred here even though it is less well defined.

Terms such as "flu-like" illness will also be avoided since various definitions exist with varying degrees of sensitivity and specificity and are usually based on some or all of the following features: fever, headache, sore throat, cough, myalgia, nasal congestion, weakness and anorexia. The term "non-specific clinical features" is also difficult to define and inevitably rather subjective unless studies have shown that a disease can not be reliably identified from its clinical features alone. Such findings will also depend on what other diseases are present in a particular area. For example, hepatosplenomegaly may seem like a rather specific clinical feature, but it occurs in

so many different UFIs (especially in the tropics) that it may not help very much in making diagnoses.

When performing a literature review on this topic the following terms were all used :

- Undifferentiated febrile illness
- Undifferentiated fever
- Unexplained febrile illness
- Unexplained fever
- Fever of unknown/unexplained origin
- Pyrexia of unknown/unexplained origin

However, the order of the terms listed above indicates which were most useful in identifying relevant references for a fever study in the tropics (in descending order).

1.2 Significant Causes of Undifferentiated Febrile Illness in Sri Lanka

The term "significant" is somewhat subjective, but is meant to include common causes of UFI that are either life-threatening or likely to lead to hospital admissions.

1.2.1 Historical Perspective

Historically, most infectious diseases were just called "fevers" until improvements in clinical skills and investigations allowed them to be distinguished from each other. In Sri Lanka the earliest known classification of disease can be found in the masks used for the *Sanni Yakuma* exorcism ritual [Bailey & de Silva, 2006] and one of these is *Gini Jala Sanniya* – the demon of high fevers (Figure 1.2.1). In 1660 a British hostage (Robert Knox) of the Kandyan king in Sri Lanka, described his surroundings as, "extraordinarily sickly by agues and feavours whereof many died" and also wrote that, "my ague came to a settled course that is one in three days and so continued for sixteen months time", which taken together are suggestive of falciparum malaria [Edirisinghe, 1988]. After taking control of Sri Lanka in 1796, British troops were plagued by high morbidity and mortality rates due to "jungle fever" [Marshall, 1846] until Major Ronald Ross IMS discovered the role of the mosquito in malaria

transmission in 1897 and control measures were instituted [Edirisinghe, 1988]. This is just one of many examples where UFIs have affected military deployments and the diseases responsible have been a problem for British expeditionary forces ever since the time of the Crusades [Bailey *et al.*, 2011].

Figure 1.2.1. *Gini Jala Sanniya* – the demon of high fevers

1.2.2 Malaria

Malaria is a systemic infection of erythrocytes caused by *Plasmodium* protozoa that are usually transmitted by the bite of *Anopheles* mosquitoes. It is found throughout the tropics and sub-tropics wherever mosquitoes breed – except in a few areas (mostly islands) where eradication of the disease has been successful. Following transmission, there is an incubation period ranging from 10 days up to >1 year in certain circumstances [Kotwal *et al.*, 2005; Boecken & Bronnert, 2005] before illness

develops. The clinical features (symptoms & signs) of malaria are non-specific until the later stages of the disease when complications occur leading to more characteristic patterns of disease. Therefore investigations are essential for the early diagnosis of malaria and traditional techniques such as microscopy of thick and thin blood films are now being supplemented with antigen detection kits, which are especially useful when expertise or laboratory facilities are limited [Abba *et al.*, 2011]. Early treatment of malaria is important to minimise morbidity and mortality and requires the use of quinine or artemisinin-based drugs with supportive therapies in complicated cases and also follow-up eradication therapy for hypnozoites of certain species [Lalloo *et al.*, 2007; World Health Organisation, 2000]. Death from various complications and long-term morbidity from cerebral complications may occur. Preventative measures are based on bite avoidance, vector control, prompt diagnosis & treatment of cases and also chemoprophylaxis for travellers.

In Sri Lanka, malaria seems to have been present since the end of the 13th century AD, but probably not before this date [Edirisinghe, 1988]. Major epidemics wee reported during the period of British colonial rule and the most severe of these was in 1934-5 when ~100,000 people died [Gill, 1936]. From 1947-1963 malaria was almost eradicated by dichlorodiphenyltrichloroethane (DDT) spraying, but case numbers then increased again due to DDT resistance in mosquitoes and internal conflict that interrupted the eradication programme [Edirisinghe, 1988]. Despite a switch to malathion spraying in 1973 and improved case detection and treatment from 1977, morbidity levels in 1986 were as high as in 1952 [Pinikahana & Dixon, 1993]. However, since 2000 the improving security situation in malarious areas and subsequent enhancement of the eradication programme has led to dramatic benefits [Briët *et al.* 2005]. Malaria control has now entered the pre-elimination stage with very few indigenous cases being reported [Rajakaruna *et al.*, 2010; Fernando *et al.*, 2009]. Not surprisingly, studies of malaria in Sri Lanka have shown that it had a bi-modal occurrence each year associated with rainfall [Briët *et al.*, 2008] and that clinical diagnosis of cases was not reliable [van der Hoek *et al.*, 1998]. Today malaria in Sri Lanka is still diagnosed by microscopy of thick and thin blood films because there remains widespread expertise with this technique and hence no need for the use of malaria antigen kits.

1.2.3 Enteric Fever

Enteric fever is a systemic infection caused by *Salmonella typhi* and *S. paratyphi* bacteria that are usually transmitted faeco-orally and then infect epithelial cells and mononuclear phagocytes. It was previously found in temperate and tropical countries, but is now restricted to developing countries due to successful control programmes elsewhere. Following transmission and an incubation period ranging from 6 to 30 days, the disease normally has an insidious onset with worsening fever and non-specific clinical features in the first week followed by abdominal pain and possibly hepatosplenomegaly, lymphadenopathy or "rose spots" in the second week. If untreated then diarrhoea and many different organ-specific features may occur in the third week including cholecystitis, hepatitis, urinary tract infection (UTI), pneumonia, meningitis, neuropathy, cardiomyopathy, osteomyelitis, haemolysis, thrombo-embolism and intestinal haemorrhage or perforation. If the patient survives then recovery will usually begin by the fourth week. Paratyphoid infection is often thought to produce a less severe illness with minimal mortality compared to typhoid infection, but recent studies have described indistinguishable clinical syndromes for the two diseases [Maskey *et al.*, 2006].

Although the clinical features of enteric fever become more characteristic as the disease progresses, it is important to give specific anti-microbial treatment at an early stage to limit its morbidity and mortality and so clinical prediction rules or early laboratory diagnosis are important. Clinical prediction rules to distinguish enteric fever from other causes of fever elsewhere in Asia have been unsuccessful and the same study also found that typhoid and paratyphoid were indistinguishable [Vollaard *et al.*, 2005]. A study in Turkey did develop a validated clinical prediction rule for identifying enteric fever using culture positive cases only [Hosoglu *et al.*, 2006], but this was specific to an area that lacks the most common causes of undifferentiated febrile illness in Sri Lanka and it also included use of the Widal serology test, which is unreliable when used on single samples in the early stages of the disease or in vaccinated individuals. An earlier study in Malaysia developed an unvalidated prediction system [Ross & Abraham, 1985], but this included serology-positive, culture-negative enteric fever cases and also relied on Widal test results. Therefore laboratory diagnosis remains most important and since anti-microbial resistance is a

major problem in enteric fever, this means that microbiology cultures of blood, faeces, urine or bone marrow remain the gold standard diagnostic tests [Parry *et al.*, 2011]. The Widal serology test is simple, quick and cheap to perform, but suffers from non-standardised reagents and inappropriate interpretation of results, which lead to a low sensitivity and specificity overall. Other serology tests based on the *S. typhi* Vi antigen produce more accurate results, but their usefulness in the acute situation is limited by the need for paired (acute and convalescent) samples. Several attempts have been made to package serology tests for enteric fever into rapid diagnostic (point-of-care) tests, but these remain insufficiently sensitive, specific and consistent to be of use in endemic countries [Parry *et al.*, 2011]. The development of antigen detection and nucleic acid amplification tests to diagnose enteric fever remains a challenge, but these do promise to overcome many of the inherent limitations of serology tests. The widespread development of fluoroquinolone resistance in *S. typhi* and *S. paratyphi* means that anti-microbial treatment with intravenous (IV) ceftriaxone or oral (PO) azithromycin is required, which has implications for costs and the further development of resistance if their use is not targeted appropriately [Butler, 2011]. Prevention of enteric fever is based on good hygiene and sanitation measures, adequate treatment of cases and long-term carriers and also vaccination, which has a cumulative efficacy of only 51-55% over 3 years [Fraser *et al.*, 2007] and is ineffective against most paratyphoid infections.

In Sri Lanka the number of enteric fever notifications reduced gradually from 2987 cases in 2004 to 1805 cases in 2007 and these were predominantly from the northern parts of the country affected by internal conflict [Epidemiology Unit, 2004-2007]. Most of these diagnoses would have been made without laboratory investigations and overall the disease is thought to be relatively uncommon in Sri Lanka compared to other more densely populated south Asian countries [de Silva & Premaratna, personal communication]. However, confirmed enteric fever does seems to occur in sufficient numbers in Sri Lanka to produce several case reports, including some that describe chloramphenicol and ciprofloxacin resistance [de Silva *et al.*, 1993; Karunanayake & Atukorala, 2004] and also a case series on ileal perforation [Peiris *et al.*, 1993a] and an evaluation of a new serology test using 29 culture-positive cases [Herath, 1993].

1.2.4 Other Bacteraemias

If blood cultures are performed to look for enteric fever organisms in undifferentiated febrile illnesses, then it is likely that other bacteraemias resulting from occult infections will also be discovered. Examples include endocarditis, lower respiratory tract infections (LRTIs), intra-abdominal collections and urinary tract infections (UTIs), which sometimes lack their distinctive clinical features. Patients who are immunocompromised may also develop systemic infections without focal features due to organisms that would normally be better contained by the immune system.

Melioidosis is a rare systemic infection caused by *Burkholderia pseudomallei* bacteria that are transmitted from soil or water through skin wounds or inhalation. The disease and organism responsible were first described in opium addicts in Rangoon by Capt Alfred Whitmore IMS [Whitmore & Krishnaswami, 1912] and it is now known to occur throughout south and south-east Asia with known risk factors including rice paddy farming and diabetes mellitus. Melioidosis usually begins after an incubation period of 1 to 21 days, but latent infection with recurrence many years later has also been described. A minority (~25%) of patients will present with an undifferentiated febrile illness, whilst the majority (~75%) will present with fever and focal clinical features due to pneumonia, septic arthritis, osteomyelitis, cellulitis, intra-abdominal abscesses or other localised infections. Diagnosis relies on standard blood cultures with careful identification of isolates and serology tests that are not widely available. Treatment usually begins with IV ceftazidime or an IV carbapenem for at least 10-14 days and is then usually followed by co-trimoxazole and doxycycline for 12-20 weeks to prevent recurrence [Limmathurotsakul & Peacock, 2011]. This regimen is expensive and difficult to deliver in developing countries and surgical intervention may also be necessary. Prevention measures are limited to occupational advice at the present time. In Sri Lanka melioidosis has been occasionally reported for many years and the organism responsible has also been found in soil samples [Inglis *et al.*, 2008].

Other bacteria that cause undifferentiated febrile illnesses, but which do not grow in standard blood cultures include those responsible for brucellosis, tuberculosis, leptospirosis and Q fever.

1.2.5 Brucellosis

Brucellosis is a systemic infection caused by *Brucella* species of bacteria that are usually transmitted from infected animals through ingestion, inhalation or skin abrasions and then infect cells of the mononuclear phagocyte system. The organism responsible was first described in British soldiers in Malta by Capt (later Major-General Sir) David Bruce RAMC [Bruce, 1887] and the disease is now mostly found in developing countries with limited veterinary resources. Following transmission and an incubation period ranging from 5 days to several months, the disease usually has an insidious onset with fever and non-specific clinical features initially. Thereafter more specific features may be lymphoid (hepatosplenomegaly or lymphadenopathy), osteoarticular (sacroiliitis, spondylitis, septic arthritis or osteomyelitis), genitourinary (epididymo-orchitis, spontaneous abortion, glomerulonephritis, abscesses), neurological (meningo-encephalitis or neuropathy) or in other sites.

This variety of presentations makes clinical diagnosis difficult and so laboratory confirmation is needed by specialist extended blood cultures, which have low yields in chronic cases or serology tests, which include some promising rapid point-of-care tests [Franco *et al.*, 2007]. Combination treatment with doxycycline (for 6 weeks), rifampicin (for 6 weeks) and gentamicin (for 2 weeks) seems to offer higher cure rates than earlier WHO regimens [Skalsky *et al.*, 2008; World Health Organisation, 2006]. Prevention of this zoonotic infection relies on occupational advice and veterinary measures including vaccination, but no human vaccine or chemoprophylaxis is available.

In Sri Lanka human brucellosis has been occasionally reported for many years [Maretic *et al.*, 1962; de Silva & Wijeratne, 1981] and seroepidemiology studies in animals have shown ~5% seroprevalence in cattle, mostly from the eastern and northern (dry zone) parts of the country [Sixl *et al.*, 1988; Silva *et al.*, 2000].

1.2.6 Tuberculosis

Tuberculosis (TB) due to *Mycobacterium tuberculosis* bacteria usually occurs as a localised respiratory infection, but ~50% of cases have extra-pulmonary features and the disease may present as an undifferentiated febrile illness – especially in those who are immunocompromised by drugs or other diseases such as HIV infection. The disseminated or miliary form of TB usually presents with fever and non-specific clinical features except for possible lymphadenopathy, hepatosplenomegaly or nodular shadowing on chest radiographs. In such cases a clinical diagnosis is necessary initially, but this may be confirmed later by specialist cultures of blood, lymph node biopsies or early morning urine samples. Tuberculin skin testing (TST) and TB interferon-gamma release assays (IGRAs) can also be useful in diagnosis, but may not be able to distinguish between active and latent disease. Treatment requires multi-drug therapy with drugs such as rifampicin, isoniazid, pyrazinamide and ethambutol in regimens that last for at least 6 months overall and there are currently major concerns regarding the development of multi-drug resistance. Preventative measures may include Bacille Calmette-Guérin (BCG) vaccination, but this is of limited efficacy and so prompt isolation, diagnosis and treatment of cases is essential.

In Sri Lanka TB is less common than in other more densely populated south Asian countries with a prevalence of 79 per 100,000 population and an incidence of 27 per 100,000 population for new smear positive cases [WHO, 2008]. Although the number of cases is relatively small, the socio-economic impact of having TB for individuals and their families is at least 3-4 times greater than for other communicable diseases in Sri Lanka [Morton-Bailey, 2010]. The Central Chest Hospital, Welisara in Gampaha District is the specialist centre for TB in Sri Lanka, but due to limited resources they do not usually attempt cultures from "clinically-obvious" TB cases and this is reserved for cases where the diagnosis is less certain or drug resistance is suspected. Isolates from recurrent TB cases at this hospital have been studied and 46% had resistance to at least one drug with individual drug resistance rates varying from 7.6% for isoniazid to 15.3% for p-amino salicylic acid (PAS) [Magana-Arachchi *et al.*, 2010].

1.2.7 Leptospirosis

Leptospirosis is a systemic infection caused by *Leptospira* spirochaetes (spiral-shaped bacteria) that are usually transmitted from infected animals through direct contact with their urine or indirectly when contaminated freshwater or wet soil comes into contact with skin or mucous membranes (including ingestion). There are currently 13 pathogenic species and an overlapping classification based on >260 different serovars (serotypes) and also additional saprophytic species and serovars [Adler & de la Peña Moctezuma, 2010]. This makes the epidemiology of leptospirosis difficult to study, but it is known that nearly all mammals can become infected and some (*eg.* rodents) act as carriers due to chronic infection of the proximal renal tubules. Leptospirosis is usually said to be the most widespread zoonotic disease in the world and is more common in developing countries and certain high-risk activities (*eg*. farming, water sports).

Following transmission the spirochaetes circulate to all organs and cause endothelial damage, but asymptomatic infection is common and only 10% of symptomatic infections lead to severe (icteric) disease. After an incubation period ranging from 2 to 26 days, the disease usually has an abrupt onset with fever and non-specific clinical features initially. Headache, myalgia and muscle tenderness in the lower trunk and legs may be severe, but conjunctival suffusion is a more specific early finding. Thereafter other more specific features may include oliguria, acute renal failure, icterus, hepatitis, (haemorrhagic) pneumonitis, haemorrhage, meningitis, myocarditis and uveitis. A biphasic febrile illness consisting of a "leptospiraemic phase", temporary recovery and then an "immune phase" is sometimes described, but is not a consistent finding. The diagnosis of leptospirosis can occasionally be made by dark-field microscopy (DFM) or specialist cultures of urine or blood, but the former is very insensitive and the latter take 2-4 weeks to become positive [Wuthiekanun *et al.*, 2007]. Serology tests remain the main methods used, but these are complicated due to the large number of different serovars that can cause infections and are usually negative at presentation [Limmathurotsakul *et al.*, 2012]. Diagnostic PCR tests have been developed, but are likely to be of limited value in developing countries [Thaipadungpanit *et al.*, 2011; Bourhy *et al.*, 2011]. Treatment

of leptospirosis with doxycycline for mild disease or IV benzylpenicillin for severe disease is recommended and should be given as early as possible. Prevention of this zoonotic infection relies on occupational advice, environmental health measures and chemoprophylaxis with doxycycline, but no vaccine is available.

In Sri Lanka leptospirosis was first identified in 1953 and in 1962 past or present infection was found in 26 (24%) of 109 patients in a fever study and in 19 (12%) of 163 people surveyed in high-risk areas [Maretic *et al.*, 1962]. In 1964 a clinical study of 63 confirmed acute cases seen at the Colombo General Hospital showed marked seasonal variation coinciding with the rainy seasons in May and October and that more cases were from the north Colombo area than central or southern parts [Rajasuriya *et al.*, 1964]. This study also described the frequency of clinical and laboratory findings in detail (Table 1.2.7.1), but there was selection bias towards more severe cases and even then the most common individual findings could nearly all have been due to other infections that cause undifferentiated febrile illnesses in Sri Lanka. Nevertheless the authors felt that the combination of fever with myalgia and conjunctival suffusion was sufficient to make a diagnosis in most cases. In 1974 a similar study at the Colombo North General Hospital also targeted suspected leptospirosis cases only, but recruited 81 patients with a wider range of presentations [Ramachandran *et al.*, 1974]. The clinical and laboratory findings were similar (Table 1.2.7.2) and fever, myalgia and conjunctival suffusion were present in >80% of cases. From 1974 to the beginning of the Ragama Fever Study the only original scientific publication on leptospirosis in Sri Lanka was a single case report. The frequencies of leptospirosis cases notified in Sri Lanka and the Gampaha District before and during the Ragama Fever Study are shown in Table 1.2.7.3 [Epidemiology Unit, 2004-2007], but most of these will be clinical diagnoses only.

Table 1.2.7.1. Clinical and laboratory features of leptospirosis in Ceylon (1964)

History	%	Examination	%	Investigations	%
Fever	100	Fever	100	↑ ESR	100
Myalgia	97	Conjunctival suffusion	95	Positive serology	96
Male sex	95	Icterus/jaundice	60	Neutrophilia	94
Headache	91	Hepatomegaly	44	↑ Urea	73
Sudden onset	87	Pneumonitis	35	Albuminuria	70
Chills / rigors	81	Haemorrhage	14	↑ Bilirubin	66
Nightmares	64	Bradycardia	14	Positive DFM of blood	40
Insomnia	56	Hypotension	14	Positive DFM of urine	35
Nausea/vomiting	56	Neurological signs	13	↑ Liver transaminases	35
Constipation	38	Lymphadenopathy	10		
Arthralgia	27	Neck rigidity	5		
Photophobia	23	Splenomegaly	3		
Delirium	16				
Deafness/tinnitus	10				
Diarrhoea	8				
Death	3				

Table 1.2.7.2. Clinical and laboratory features of leptospirosis in Sri Lanka (1974)

History	%	Examination	%	Investigations	%
Male sex	99	Fever	98	Positive serology	95
Fever	98	Conjunctival suffusion	86	↑ ESR	85
Myalgia	93	Monophasic fever	83	Albuminuria	57
Death	10	Icterus/jaundice	49	↑ Urea	51
		Hepatomegaly	42	Leukocytosis	50
		Oliguria/anuria	22	↑ Bilirubin	49
		Hypotension	21	↑ Liver transaminases	35
		Meningism	8	Positive blood culture	4

Table 1.2.7.3. Leptospirosis Cases in Sri Lanka and Gampaha District (2004-7)

Leptospirosis	Total Cases in Sri Lanka	Cases in Gampaha (%)	Gampaha *vs* Other Districts
2004	1427	133 (9%)	=4th
2005	1537	220 (14%)	1st
2006	1568	211 (13%)	2nd
2007	2187	311 (14%)	1st

1.2.8 Q Fever

Q fever is a systemic infection caused by *Coxiella burnetti* bacteria that are usually transmitted from infected animals through direct contact or inhalation of an environmental spore-like form, which then infect cells of the mononuclear phagocyte system. Following an incubation period ranging from 1 to 60 days, the disease usually has an acute onset with an undifferentiated febrile illness, atypical pneumonia or anicteric hepatitis. Acute Q fever is usually self-limiting after a few weeks, but chronic Q fever infection may develop months to years later in 1-5% of cases and 68% of these are due to endocarditis with the remainder presenting as other chronic vascular infections, respiratory infections, undifferentiated febrile illnesses, hepatitis or osteomyelitis [Brouqui *et al.*, 1993]. The clinical features of Q fever are non-specific and so laboratory diagnosis is essential. This is based on serology tests and also PCR tests that have only recently become established. Q fever serology measures antibody responses against *C. burnetti* Phase 2 antigens (which are raised in acute Q fever) and Phase 1 antigens (which are raised in chronic Q fever) and the definition of positive results varies between different laboratories [Healy *et al.*, 2011]. Treatment of acute Q fever with doxycycline 200 mg/day for 14 days seems to reduce morbidity [Dijkstra *et al.*, 2010], but chronic Q fever usually requires treatment with doxycycline and hydroxychloroquine for at least 18 months [Raoult *et al.*, 1999; Million *et al.*, 2010]. Prevention of this zoonotic infection relies on occupational advice and veterinary measures including vaccination and prevention in humans can also be achieved with vaccination [Gidding *et al.*, 2009] or chemoprophylaxis [Benenson & Tigertt, 1956].

In Sri Lanka Q fever has been reported in a single clinical case [Schmid *et al.*, 1952a] and seroepidemiology studies have shown previous exposure in 4% of slaughter-house workers and from 0% to 8% of slaughtered animals [Schmid *et al.*, 1952b; Sixl *et al.*, 1988].

1.2.9 Rickettsial Infections

Rickettsial infections include the typhus fevers (louse-borne/epidemic due to *Rickettsia prowazekii* and flea-borne/endemic/murine due to *R. typhi*), spotted fevers (numerous tick-borne infections including Rocky Mountain, Mediterranean, African and Indian varieties due to numerous local species), scrub typhus (mite-borne due to *Orientia tsutsugamushi*) and also less common diseases such as bartonellosis, ehrlichiosis and anaplasmosis [Parola *et al.*, 2005; Botelho-Nevers & Raoult, 2007]. These primitive bacteria infect endothelial cells and following incubation periods from 2 to 16 days (or longer for bartonellosis), the diseases usually have an acute onset with an undifferentiated febrile illness. Thereafter skin manifestations such as a centripetal rash or an eschar at the site of the arthropod bite may develop as well as multi-organ disease and death rates vary from 10-20% for epidemic typhus, ~4% for endemic typhus, and 1-35% for scrub typhus [Botelho-Nevers & Raoult, 2007]. Clinical diagnosis is limited in the early stages, especially when skin manifestations are minimal and laboratory diagnosis is also usually restricted to serology tests that are only available in specialist laboratories and suffer from cross-reactivity problems [La Scola & Raoult, 1997]. The use of the Weil-Felix serology test is no longer recommended due to poor sensitivity and specificity [Kularatne & Gawarammana, 2009] and there are no established rapid diagnostic tests available. Treatment of rickettsial infections usually consists of doxycycline given for 7-10 days depending on the species involved. Prevention relies on bite avoidance, vector control and chemoprophylaxis with doxycycline can also be given.

In Sri Lanka rickettsial infections were first reported in 1940 and in 1944 an outbreak of scrub typhus affected >750 Commonwealth troops of whom 85% had eschars and ~2% died [Sayers & Hill, 1948]. In 2001 a one-year case series from the Central Province reported 8 cases of scrub typhus, 10 cases of spotted fever and 2 cases of

murine typhus [Kularatne *et al.*, 2003]. In 2003 a one-year case series from the Western Province reported 19 cases of scrub typhus, 8 cases of spotted fever and no cases of murine typhus [Premaratna *et al.*, 2008]. Nationally, the number of notified rickettsial infections increased from 789 cases in 2005 to ~1,200 cases in subsequent years and most of these were from the central, southern and northern parts of the country [Epidemiology Unit, 2005-7].

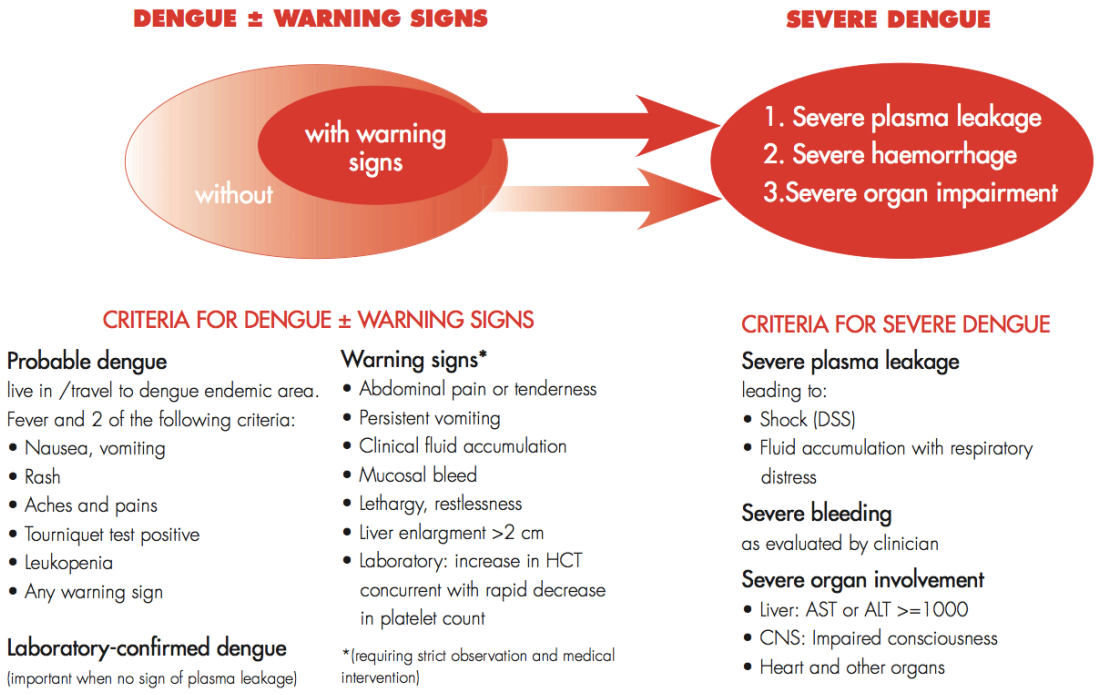
1.2.10 Dengue

Dengue is a systemic infection caused by dengue virus (DENV), which is a flavivirus transmitted by the bite of *Aedes* mosquitoes that usually breed in urban areas. There are 4 serotypes of dengue virus (DENV-1, DENV-2, DENV-3 & DENV-4). Humans can be infected once with each of these and are the only known reservoir of infection. Dengue is the most common arbovirus infection worldwide and is found in most tropical areas with the potential to cause outbreaks and spread wherever appropriate *Aedes* mosquitoes are found. Following transmission dengue virus infects mononuclear phagocyte cells and may lead to a sub-clinical illness or after an incubation period of 3-14 days the disease presents with an acute onset of either classic dengue fever (DF), dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS).

Dengue typically presents with an undifferentiated febrile illness of the "fever-arthralgia-rash" type and may include features such as headache, retro-orbital pain, arthralgia, myalgia, nausea, vomiting, a maculopapular rash that can become confluent with "islands of sparing", petechiae, leukopenia and thrombocytopenia. The illness persists for 2-7 days, sometimes with a biphasic fever and then settles spontaneously with an overall mortality of 1%. DHF and DSS mostly occur in patients who have previously been infected by another dengue virus serotype and are thought to be due to a process of "antibody-dependant immune enhancement" that leads to more serious illnesses [Halstead, 2007]. DHF is defined as a combination of fever (for 2-7 days), haemorrhage (positive tourniquet test, petechiae, ecchymoses, purpura, bleeding from mucosa, gastrointestinal tract or injection sites), thrombocytopenia (<100,000 cells/mm^3 or <100 x 10^9/L) and plasma leakage (pleural effusion, ascites, hypoproteinaemia, haematocrit >20% above normal or

>20% decrease after fluid resuscitation). DSS is defined as DHF with circulatory failure (cool peripheries with sweating and restlessness, tachycardia with low volume pulse, hypotension or pulse pressure <20 mm Hg). These definitions were established in previous WHO guidelines [WHO, 1997] and were designed more for research than clinical purposes. Therefore the most recent guidelines (published after the Ragama Fever Study was performed) are much simpler and use the term "severe dengue" to include both DHF and DSS [WHO, 2009]. A summary of the latest definitions is shown in Figure 1.2.10 and these continue to be evaluated at present.

Figure 1.2.10. WHO dengue case classification and levels of severity (2009)



The haemagglutination inhibition (HAI) test and plaque reduction neutralisation technique (PRNT) are still officially the "gold standards" for diagnosing dengue [WHO, 1997]. The HAI test has good sensitivity and PRNT is specific enough to distinguish different serotypes, but both are impractical and no longer used for diagnosis [WHO, 2004]. For this they have been replaced by capture ELISAs for IgM and IgG antibodies and also reverse transcriptase polymerase chain reaction (RT-PCR) tests, which have good sensitivity and specificity respectively [Peeling *et al.*, 2010]. However, the former do suffer from cross-reactivity with other flaviviruses (including yellow fever and Japanese encephalitis) and the latter are not

widely available in developing countries. Rapid diagnostic tests for dengue include numerous serology tests for IgM and IgG antibodies and newer "non-structural 1" (NS1) antigen detection tests [Blacksell *et al.*, 2011b]. Simultaneous testing for IgM and IgG antibodies and also NS1 antigen is necessary to achieve acceptable levels of diagnostic accuracy [Blacksell *et al.*, 2011b], but sensitivities up to 87% and specificities up to 97% can be achieved in this way [Blacksell *et al.*, 2012]. Treatment of all forms of dengue consists of supportive treatment including early and careful fluid resuscitation and the use of platelet transfusions and critical care facilities where available. Prevention currently relies on bite avoidance and mosquito control and development of a vaccine remains challenging [Konishi, 2011].

In Sri Lanka dengue was first described in the early 20th century [Mendis, 1967], but despite all 4 serotypes being present there were very few DHF/DSS cases reported until 1989, which could not be explained at the time [Messer *et al.*, 2002]. Since then there have been major outbreaks in 2002 and 2004 and these were thought to be due to changes in the circulating DENV-1 genotype and DENV-3 clades [Kanakaratne *et al.*, 2009]. An adult case series from Kandy in the Central Province in 2001-3 [Kularatne *et al.*, 2005] recruited 183 confirmed dengue cases using a serology test after one week of illness, which showed either IgM (suggestive of primary infection) or IgM and IgG antibodies (suggestive of secondary infection). Clinical and laboratory findings were similar in both confirmed cases and those that were just clinically-diagnosed, but only the former are shown here (Table 1.2.10.1). There was also a trend towards leukopenia that was most marked on the 5th day of fever. The authors suggested that a combination of the clinical picture, thrombocytopenia, leukopenia and elevated liver enzymes could be used as markers for early diagnosis of dengue infection. Another adult series from Colombo in 2004 [Malavige *et al.*, 2006] recruited 108 confirmed dengue cases using similar serology tests and criteria. Similar clinical and laboratory findings were reported (Table 1.2.10.2) although there were more DHF cases in this later series. The authors also compared clinical and laboratory features of dengue from studies in Sri Lanka, Singapore, Cuba, Thailand, Nicaragua, Bangladesh and Taiwan and found significant variation, which might have been due to a difference in the number of primary or secondary and DF or DHF cases in each series. The frequencies of dengue cases notified in Sri Lanka and the Gampaha District before and during the Ragama Fever Study are shown in Table

1.2.10.3 [Epidemiology Unit, 2004-2007], but most of these will be clinical diagnoses only.

Table 1.2.10.1. Clinical and laboratory features of dengue in Kandy (2001-3)

History	%	Examination	%	Investigations	%
Headache	79	Fever	21	Platelets <100 x 10^9/L	91
Myalgia	74	Flushing	40	Tourniquet test positive	36
Vomiting	62	Rash	15	↑ Liver transaminases	31
Male Sex	59	Haemorrhage	13		
Death	1	Effusions	3		

Table 1.2.10.2. Clinical and laboratory features of dengue in Colombo (2004)

History	%	Examination	%	Investigations	%
Myalgia	76	Hepatomegaly	45	Platelets <100 x 10^9/L	79
Headache	66	Flushing	42	WCC < 4 x 10^9/L	31
Vomiting	64	Haemorrhage	42	↑ Haematocrit	22
Male sex	59	Shock	14		
Arthralgia	57	Effusions	11		
Diarrhoea	29	Splenomegaly	<1%		
Abdominal pain	17				
Death	4				

Table 1.2.10.3. Dengue cases in Sri Lanka and Gampaha District (2004-7)

Dengue	Total Cases in Sri Lanka	Cases in Gampaha (%)	Gampaha *vs* Other Districts
2004	15414	3015 (20%)	2nd
2005	5882	1243 (21%)	2nd
2006	11902	1774 (15%)	2nd
2007	7271	980 (13%)	2nd

1.2.11 Japanese Encephalitis

Japanese encephalitis is a cerebral infection caused by Japanese encephalitis virus (JEV), which is also a flavivirus transmitted by the bite of *Culex* mosquitoes that breed in rural areas. The natural reservoirs are birds and the most important amplifying hosts are usually domestic pigs. Japanese encephalitis is found throughout south-east Asia, causes outbreaks when it reaches new areas and now extends as far as Pakistan and Australia. Following transmission there is an incubation period ranging from 5 to 15 days before the disease begins with an undifferentiated febrile illness [Misra & Kalita, 2010]. Seroepidemiology studies show that only 1 in 25 to 1 in 1000 infections lead to encephalitis [Halstead & Grosz, 1962; Huang, 1982] and the remainder have only an undifferentiated febrile illness or no symptoms at all. A study in Thailand showed that JEV infection was responsible for 22 (14%) of 156 patients hospitalised with acute undifferentiated fever and none had neurological features [Watt & Jongsakul, 2003]. Even when Japanese encephalitis does present with neurological features, these may be variable, diverse and non-specific compared to other CNS infections occurring in affected countries. Neurological features include seizures, Parkinsonian features, flaccid paralysis, upper motor neurone lesions, abnormal movements, cerebellar signs, cranial nerve palsies, abnormal behaviour and coma. Japanese encephalitis leads to death in a third of cases, long-term morbidity in a third and complete recovery in the remaining third [Gould & Solomon, 2008].

Hence laboratory investigations are required to confirm JEV infections, which consist of serology tests that can also be performed on CSF and RT-PCR tests that are not widely available in developing countries. The standard serology tests are now IgM and IgG capture ELISAs that use specific JEV antigens, but testing in parallel for dengue and careful interpretation of results is recommended due to cross-reactivity between these two flaviviruses. Anti-JEV IgM is detectable in serum and CSF in 70-75% of cases after 4 days of illness and in >95% of cases after 7-10 days of illness [WHO, 2007]. Commercial versions of these tests have been developed (including some kits that test in parallel for dengue) and these have sensitivities varying from 17 to 96.7% and specificities varying from 89 to 99.5% if dengue

serology is also performed [Jacobson *et al.*, 2007; Lewthwaite *et al.*, 2010; Khalakdina *et al.*, 2010; Robinson *et al.*, 2010].

No specific anti-viral treatment for JEV infection is available at present and so supportive therapy is all that can be offered, which may involve critical care facilities and lifelong community care to overcome the range and severity of possible neurological complications that may occur. Prevention relies on bite avoidance and mosquito control (which are difficult in rural areas) and vaccination of both domestic hosts and humans. Currently available Japanese encephalitis vaccines usually require boosters every 2-3 years and are expensive, but improved and cheaper vaccines are now being developed and licensed [Halstead & Thomas, 2011].

In Sri Lanka JEV was first isolated in 1974 and occurred sporadically until 1985 when an outbreak led to >500 cases in the northern and western parts of the country and a similar outbreak occurred in 1987 [Peiris *et al.*, 1992]. A seroepidemiology study in 1988 showed evidence of previous JEV infection in 10 to 20% of school children in Ragama, which is within the Gampaha District of the Western Province and it also had the highest recorded annual incidence of encephalitis cases (74 per 100 000) in 1990 and 1991 [Peiris *et al.*, 1993b]. Since then a childhood vaccination programme in affected areas of Sri Lanka seems to have been effective, but uncertainty remains due to limited resources for laboratory diagnosis and the fact that only 0.1 to 4.0% of JEV infections lead to encephalitis.

1.2.12 Chikungunya

Chikungunya is a systemic infection caused by chikungunya virus (CHIKV), which is an alphavirus transmitted by the bite of *Aedes* mosquitoes that usually breed in urban areas. The name of the disease means “that which bends over” in the local Makonde language following its discovery in Tanganyika in 1952 [Robinson, 1955]. There are distinct African and Asian genotypes of the virus and in Africa non-human primates are thought to be important reservoirs of infection. Periodic epidemics of chikungunya occur including one in the 1960s, which spread through several Asian countries and another that spread from Kenya around the Indian Ocean to south-east Asia in 2004-8 [Staples *et al.*, 2009]. Following transmission CHIKV infects

mononuclear phagocyte cells, endothelial cells, fibroblasts and myoblasts leading to a sub-clinical illness or else after an incubation period of 1-12 days the disease presents as an undifferentiated febrile illness of the "fever-arthralgia-rash" type.

Clinical features include fever (that may be biphasic), arthralgia (which is polyarticular and affects mostly the small joints), rash (that is maculopapular) and also headache and gastrointestinal symptoms. Its most characteristic feature is persistent arthralgia that can continue for years in some patients and there seems to be a minimal risk of mortality [Burt *et al.*, 2012]. The only reported laboratory feature was said to be leukopenia and diagnosis is based on serology tests including haemagglutination inhibition (HAI) and enzymed immunosorbent assays (EIA) for IgM antibodies and a RT-PCR test has recently been developed. Treatment of chikungunya consists of supportive treatments only and persistent arthralgia is the most difficult symptom to control. Prevention currently relies on bite avoidance and mosquito control.

In Sri Lanka an outbreak of chikungunya occurred in May-July 1965 and was found to affect mostly urban areas [Mendis, 1967]. Serum samples taken before the outbreak showed existing antibodies against chikungunya in 86% of subjects aged >40 years, but 0% of those aged <11 years suggesting that the virus had been present in Sri Lanka ~40 years previously [Hermon, 1967]. A case series from Colombo in 1965 [Munasinghe *et al.*, 1966] recruited 42 confirmed chikungunya cases using serology tests. Detailed clinical and laboratory features were reported (Table 1.2.12) and it was noted that chikungunya was difficult to distinguish from dengue, but could be distinguished from leptospirosis by the absence of nightmares and having an ESR <50 mm/hr. No cases of chikungunya were identified in Sri Lanka in the few years before the Ragama Fever Study, but in 2006 an epidemic of a new CHIKV variant was known to be spreading from east Africa around the Indian Ocean and had already reached India [Yergolkar *et al.*, 2006].

Table 1.2.12. Clinical and laboratory features of chikungunya in Ceylon (1965)

History	%	Examination	%	Investigations	%
Arthralgia	100	Fever	100	Platelets <100 x 10^9/L	74
Headache	91	Lymphadenopathy	100	ESR <40 mm/hr	96
Anorexia	83	Conjunctival suffusion	66	ESR <20 mm/hr	67
Chills	81	Relative bradycardia	54	WCC < 4 x 10^9/L	9
Flushed face	81	Rash	43	↑ Liver transaminases	3
Myalgia	69	Hypotension	30	Tourniquet test positive	2
Backache	48	Biphasic fever	17		
Vomiting	33	Bleeding	17		
Constipation	31	Meningism	10		
Sore throat	29	Splenomegaly	7		
Rigors	26	Hepatomegaly	2		
Diarrhoea	17				
Photophobia	17				

1.2.13 Hantavirus Infection

Hantaviruses from the Bunyaviridae family cause systemic infections leading to haemorrhagic fever with renal syndrome (HFRS) in Asia and Europe and Hantavirus pulmonary syndrome (HPS) in North and South America. They were probably responsible for >35,000 cases of "trench nephritis" in British troops on the Western Front during World War I [Atenstaedt, 2006] and led to >3,000 military cases of "epidemic haemorrhagic fever" during the Korean War [Paul & McClure, 1958] and also affected British troops during the last Balkans Conflict [Stuart *et al.*, 1996]. More than 21 different hantaviruses are known to live in various rodents and cause disease in humans and the most important examples are Hantaan virus (HTNV) and Seoul virus (SEOV) in Asia, Puumala virus (PUUV), Dobrava-Belgrade virus (DOBV) and Tula virus (TULV) in Europe and Sine Nombre virus (SNV) and Andes virus (ANDV) in America. The total number of pathogenic human hantaviruses may increase further with the discovery of a distinct group related to the Thottapalayam

(TPMV) hantavirus, which was originally found in insectivores (shrews) in southern India [Carey *et al.,* 1971; Song *et al.*, 2007] and is now known to extend to Nepal and China (Kang *et al.,* 2011; Guo *et al.*, 2011]. Other hantaviruses have so far only been discovered in rodents, but may cause human infection (perhaps with less obvious pathogenicity) according to serology results. Thailand virus (THAIV) and closely-related hantaviruses [Johansson *et al.*, 2010] are good examples of this in Asia [Pattamadilok *et al.*, 2006].

Hantaviruses are transmitted from their animal hosts via (aerosolised) excreta and bites and their pathogenesis in humans remains poorly understood [Jonsson *et al.*, 2010]. Following an incubation period of 14 to 28 days, the diseases usually have an acute onset with an undifferentiated febrile illness for the first 3 to 7 days followed by the more characteristic haemorrhagic, renal or pulmonary features thereafter. However, even at this stage it is clinically very difficult to distinguish hantavirus infections from similar presentations of leptospirosis. Laboratory diagnosis is based on serology tests that suffer from cross-reactivity between different hantaviruses and PCR tests that are very rarely available in developing countries. No specific anti-viral treatment is available at present and so supportive therapy for organ failures is all that can be offered. Prevention is limited to giving advice, rodent control and trying to improve the living conditions of people in affected areas.

In Sri Lanka hantavirus infection has been identified using serology tests in patients with "leptospirosis-like" infections, who remained sero-negative for leptospirosis itself. 14 (6%) of 248 patients tested had antibodies against both HTNV and SEOV hantaviruses and in 4 patients living outside Colombo there were IgM titres or high IgG titres suggestive of acute infection [Vitarana *et al.*, 1988]. A parallel study showed that 13 (14%) of 96 rats in Colombo harbour were seropositive for SEOV with lower titres against HTNV and PUUV and a hantavirus with some antigenic similarity to SEOV was isolated from them [Vitarana *et al.*, 1988]. These results suggest that novel hantaviruses may be present in Sri Lanka and that the findings in humans and rats may not be related. Similar results were found in subsequent years [Vitarana, 1994] and in southern India a more recent serotyping study showed reactivity to both HTNV and THAIV hantaviruses, but not SEOV or PUUV and TPMV testing was not performed [Chandy *et al.*, 2009a]. All these serology results

are difficult to interpret due to the genetic diversity and cross-reactivity of hantaviruses and also the possibility of novel hantaviruses being involved. However, more direct evidence comes from a related study of 344 patients with acute undifferentiated febrile illnesses, of which 18 (5%) were diagnosed with acute hantavirus infection by serology and 6 of these were also PCR positive [Chandy *et al.*, 2009b]. The sequences obtained showed 84-99% homology and were most closely related to HTNV (84-99% homology), then SEOV (69-79% homology) and THAIV (61-72% homology) and were quite distinct from TPMV (8-30% homology).

1.2.14 Other Virus Infections

Many other virus infections can present with undifferentiated febrile illnesses, including Epstein-Barr virus (EBV), cytomegalovirus (CMV), human immunodeficiency virus (HIV), some respiratory viruses (including influenza) and also polio, measles, mumps, rubella, varicella and hepatitis viruses in their initial stages. Those with the potential to cause outbreaks in developing countries have been studied in detail, but limited resources mean that diagnostic tests are only routinely performed for blood-borne viruses with serious consequences, such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV).

In Sri Lanka outbreaks of measles, mumps, varicella and hepatitis A have occurred in recent years due to conflict, lack of vaccination and relatively low seroprevalence rates for protective antibodies. Influenza outbreaks occur on a seasonal basis in Sri Lanka and were responsible for 11% of all acute respiratory tract infections seen at the Colombo North Teaching Hospital, Ragama in Gampaha District [Perera *et al.*, 2010]. There is a low incidence of chronic blood-borne virus infections in Sri Lanka with rates of <0.1% for HIV [UNAIDS, 2009], 2.5% for HBV [Padmasiri *et al.*, 1995] and 0.16% for HCV [Senevirathna *et al.*, 2011] and the country now has universal HBV vaccination with >98% coverage [WHO, 2010].

1.2.15 Non-Infectious Diseases

Several non-infectious diseases can present with undifferentiated febrile illnesses and the relative proportion of these will increase if the normal FUO/PUO definition is

used because this requires a fever duration of >3 weeks [Petersdorf & Beeson, 1961] and most infectious causes will last for a shorter time. Examples include several malignancies and autoimmune diseases, which are known to occur in the tropics.

1.3 Previous Fever Studies in South & South-East Asia

The causes of undifferentiated febrile illnesses in Sri Lanka are likely to be similar to those in other south Asian countries (*eg.* India and Nepal) and also south-east Asian countries (*eg.* Thailand, Burma and Vietnam). Therefore previous fever studies from both these regions were reviewed.

1.3.1 Sri Lanka

The only wide-ranging fever study in Sri Lanka was published >40 years ago [Maretic *et al.*, 1962]. Case definitions were not provided, but the target population seems to have been agricultural workers mostly from Western Sri Lanka who presented with febrile illnesses ("PUO") to various hospitals and had serum samples sent to the Medical Research Institute in Colombo. This seems to have been a laboratory-based study and no clinical details were recorded, but serology tests for brucellosis, leptospirosis, Q fever, rickettsial infections and arbovirus infections were performed. Its results show that in 1957 there were 225,804 cases of PUO reported in Sri Lanka with 3,644 deaths and the serology results are summarised in Table 1.3.1.

Table 1.3.1. Causes of PUO in Ceylon (1957)

Disease	Number Positive	Number Tested	Percentage Positive
Brucellosis	24	551	4%
Leptospirosis	26	109	24%
Q fever	4	27	15%
Scrub typhus	33	Not reported	-
Murine typhus	166	Not reported	-

There are obvious limitations to this study and one must assume that malaria had already been tested for at the referring hospital since it was such a common problem at the time. It is also likely that the "murine typhus" category includes cases of rickettsial spotted fevers since these could not be distinguished using the tests available at that time. Overall, it is difficult to draw any firm conclusions from this study, but it does show that the infections listed occurred in significant numbers in Sri Lanka in 1957.

1.3.2 India

The only recent published fever study from India was of "non-malarial acute undifferentiated fevers" [Joshi *et al.* 2008]. On this occasion malaria cases were excluded because these were relatively easy to diagnose using laboratory tests and there was concern that other infections causing acute undifferentiated febrile illnesses were being incorrectly diagnosed and treated as malaria, which is a common problem globally including Africa [Reyburn *et al.*, 2004]. This was a retrospective study that simply recruited all in-patients >12 year old who had been investigated for malaria from June to November 2006 in a rural hospital in central India. 144 (12%) of 1197 such patients were diagnosed with malaria and the remainder were not tested systematically or comprehensively for other infections. However, 47 (27%) of 176 tested had dengue, three (27%) of 11 tested had leptospirosis, two (18%) of 11 tested had acute hepatitis E virus infection, 12 (13%) of 90 tested had TB meningitis, four (10%) of 41 tested had acute or chronic HBV infection, seven (8%) of 90 tested had bacterial meningitis and 19 (8%) out of 240 tested had positive blood cultures, but these were mostly Gram positive contaminants and no *Salmonella* species (including those causing enteric fever) were grown. Overall, it is difficult to draw any firm conclusions from this short, retrospective, non-systematic and non-comprehensive study, but the range of infections identified is of interest.

1.3.3 Nepal

Most parts of Nepal have a different climate to Sri Lanka, but a high-quality fever study was performed in Kathmandu from January to March and July to August 2001 [Murdoch *et al.*, 2004]. All adults (≥14 years old) with fever (axillary temperature

≥38°C) that arrived at Patan hospital were invited to join the study within 24 hours of admission. After providing written informed consent, the patients were interviewed and examined with data recorded on standardised assessment forms. The information collected included demographics, symptoms, past medical history, recent antimicrobial therapy and clinical signs. Venous blood samples were collected for culture and acute serology tests and urine samples were collected for antigen tests and simple assays of anti-microbial activity [Liu *et al.*, 1999]. Patients were also asked to return 5-7 days after presentation to provide another blood sample for convalescent serology tests. Using the results obtained, multivariable logistic regression was performed to evaluate variables associated with diagnoses.

Infectious causes of fever (including some co-infections) were found in 323 (37%) of 876 patients recruited as summarised in Table 1.3.3.1. 164 patients (19%) reported taking anti-microbials before attending hospital, but urinary anti-microbial activity was found in 307 (38%). The correlation between clinical diagnoses made by the physicians and laboratory diagnoses was very poor and the logistic regression identified only a few significant associations as shown in Table 1.3.3.2.

Table 1.3.3.1. Diagnosis of febrile illnesses in Kathmandu, Nepal (2001)

Disease	**Cases***	**Percentage**
Enteric fever	117	13%
S. typhi	(60)	(7%)
S. paratyphi	(57)	(6%)
Murine typhus	97	11%
Pneumococcal pneumonia	53	6%
Leptospirosis	36	4%
Scrub typhus	28	3%
HIV infection	11	1%
Legionellosis	2	< 1%
Dengue	0	0%
Malaria	0 (of 506 tested)	0%

* N = 876

Table 1.3.3.2. Independent predictors of diagnoses in Kathmandu, Nepal (2001)

Diagnosis	Variable	OR (95% CI)	P Value
Enteric fever	Hospital admission	0.1 (0.04–0.5)	0.001
	Leukopenia	3.5 (1.6–4.7)	< 0.001
Pneumococcal pneumonia	Leukocytosis	2.2 (1.1–4.3)	0.019
	Clinical chest signs	4.4 (2.1–9.1)	< 0.001
	Radiological chest signs	3.1 (1.5–6.3)	0.002
Scrub typhus	Anaemia	17.2 (1.2–246.0)	0.04

This was a prospective, systematic study that extended across the two main seasons in Nepal and although it was focused on blood stream infections, several other important causes of febrile illness were tested for as well. Only 13% of patients returned to give convalescent blood samples at a median time of 5 days following admission and this is likely to have reduced the number of cases identified by serology tests (*eg.* murine typhus, leptospirosis, scrub typhus and dengue). Despite the logistic regression findings, the authors stated that no clinical predictors were identified to reliably distinguish between the different infections and so they recommend that the development of cheap and accurate diagnostic tests would be most appropriate in this setting.

A subsequent retrospective fever study that also concentrated on blood stream infections was performed at Dhulikhel hospital in Kathmandu from 2002-4 [Sharma *et al.*, 2006]. The medical and laboratory records were reviewed for all patients (of all ages) who presented with an axillary temperature ≥38°C and had a blood culture taken. Positive blood cultures were found in 122 (7%) of 1774 patients who met the recruitment criteria and the most common diagnoses made were enteric fever in 52% (41% typhoid and 11% paratyphoid) and pneumococcal infection in 12%. Although clinical features were recorded, they were not correlated to the final diagnoses and overall this study added little to what was already known. However, a later study performed retrospective IgM serology tests on paired samples from 103 of these same cases and made diagnoses of murine typhus in 26%, scrub typhus in 22%, leptospirosis in 10% and dengue in 8% [Blacksell *et al.*, 2007a]. Overall 18% had

evidence of co-infections and in many cases (including all of the dengue cases) the IgM titre did not increase between samples suggesting that the infections were not acute. Again no clinical correlation was provided and so the results only reinforced what was already known about the differential diagnosis of febrile illnesses in the Kathmandu area of Nepal.

1.3.4 Thailand

More fever studies have been produced in Thailand than any other country in this region. In 1991 a study of acute undifferentiated fevers was performed at Chiangrai hospital in north Thailand to determine what proportion were due to JEV infection [Watt & Jongsakul, 2003]. This prospective study was carried out during the rainy season of June to December since cases were known to be most common at that time. The patients to be recruited were defined as those *"attending the adult out-patients department ... with an oral temperature ≥37.5°C, a history of fever lasting ≤2 weeks, no known chronic medical problems, no known current infectious disease and no neurological signs"*. A standardised data collection sheet was used to collect clinical and laboratory data on each case and IgM serology for dengue and JEV infection were performed on admission samples only. From 156 patients recruited there were 35 (22%) with dengue, 22 (14%) with JEV infection and the remaining 99 (63%) were undiagnosed. However, positive IgM serology only indicates that acute infection had occurred within the last 6 weeks and testing only a single sample for each patient led to equivocal results in 8 (36%) of the JEV infection cases. Limited attempts were made to correlate clinical and laboratory features with the diagnoses due to the small numbers of cases identified. Overall, this was a prospective, standardised study that was performed at a specific time of year to identify only two specific infections. Although limited in its scope and by the serology testing performed, it remains a well-designed study and confirmed that JEV infection can present with an acute undifferentiated febrile illness.

The same authors later conducted another study at three hospitals in north, west and south Thailand to investigate the use of a combined rapid diagnostic test for scrub and murine typhus, leptospirosis, dengue and typhoid [Watt *et al.*, 2005]. In this prospective study patients were recruited if they presented with *"acute febrile*

illnesses of unclear etiology … if their duration of fever by history was <3 weeks, a blood smear for malaria was negative, and informed written consent was given." Confirmed diagnoses were made using blood, urine and stool cultures for enteric bacteria and paired IgM and IgG serology tests. Only acute sera were tested with the rapid diagnostic test under investigation. From 310 patients recruited, there were only 36 (12%) with confirmed, single diagnoses covered by the rapid diagnostic test, of whom 18 (50%) had scrub typhus, six (17%) had murine typhus, six (17%) had leptospirosis, four (11%) had dengue and two (6%) had typhoid. Results for the rapid diagnostic test were positive for the correct infection as defined by culture or serology in 94% of patients, but were also positive for incorrect infections in 92% of these cases. Overall, this was another prospective, standardised study that was conducted for the specific purpose of evaluating a new diagnostic test. Although it was a well-designed study, the frequencies of confirmed diagnoses can not be considered representative of the study group nor the wider population and the rapid diagnostic test had insufficient specificity to be of any value in this context.

A high-quality fever study was performed at a hospital near the River Kwai on the Thai-Burmese border from 1999-2002 [Ellis *et al.*, 2006]. This used a precise yet concise definition for recruitment and conduct of the study as shown :

"Adult inpatients and outpatients presenting with temperature ≥ 38°C or history of fever over the previous 48 hours were admitted to the study and evaluated for cause of fever. Patients with fever >48 hours were also eligible for enrollment, as long as the cause of fever was not yet known. Patients presenting for continuation of treatment of known cause of fever were excluded, as were those who were unable or unwilling to provide blood samples 2-4 weeks after enrollment. Clinical information and blood samples were obtained at enrollment and after approximately 3 weeks. Informed consent was obtained from each study subject before enrollment."

Routine haematology, biochemistry, renal function and liver function tests were performed for all patients at admission. Blood was also taken for malaria blood films at admission and for acute and convalescent serology tests at admission and 2-4 weeks later. Blood cultures were not performed and other investigations were performed at the discretion of the physicians responsible for patient care. From 613

patients recruited, there were 530 (86%) who returned for convalescent serology tests, 294 (48%) with laboratory diagnoses, 166 (27%) with clinical diagnoses and 153 (25%) with no diagnosis made as shown in Table 1.3.4.1. There were 34 patients (6%) with apparent dual diagnoses of whom 22 (65%) seemed to have both malaria and leptospirosis.

Table 1.3.4.1. Diagnosis of febrile illnesses on the Thai-Burmese border (1999-2002)

Diagnosis	**Cases***	**Percentage****
Malaria	155	25%
P. falciparum	(95)	(16%)
P. vivax	(48)	(8%)
Mixed or other	(12)	(2%)
Leptospirosis	107	18%
Lower respiratory infection	64	10%
Upper respiratory infection	57	9%
Rickettsiosis	36 (of 46 tested)	6%
Spotted fever group rickettsiosis	(20)	(3%)
Murine typhus	(9)	(2%)
Scrub typhus	(7)	(1%)
Gastroenteritis	16	3%
Urinary tract infection	13	2%
Dengue	9	2%
HIV/AIDS	7	1%
Pulmonary TB	7	1%
Typhoid	5 (of 31 tested)	1%
Japanese encephalitis	1	< 1%
Not diagnosed	153	25%
Other diagnoses	7	3%

* N = 613; ** Total is > 100% due to dual diagnoses

Clinical and laboratory variables that were potential predictors of specific diagnoses were analysed using univariate statistics. Variables associated with diagnoses at a

significance of $P < 0.1$ then underwent multivariate logistic regression. Variables with a significance of $P < 0.05$ after regression were considered to be independent predictors of the diagnoses. Unfortunately, the only significant predictors were for malaria as shown in Table 1.3.4.2.

Table 1.3.4.2. Independent predictors of diagnoses on the Thai-Burmese border (1999-2002)

Diagnosis	Variable	OR (95% CI)	P Value
Malaria (all)	Out-patient	3.2 (1.7–6.0)	< 0.001
	Temperature ≥38°C	2.3 (1.4–3.8)	0.001
	Platelets <150 x 10^9/L	17.2 (10.4–28.5)	< 0.001
Malaria (falciparum)	Temperature ≥38°C	2.1 (1.2–3.5)	0.006
	Platelets <150 x 10^9/L	8.8 (5.1–15.5)	< 0.001

Malaria was expected to be the most common infection in this study, but the numbers of leptospirosis cases was unexpected. The fact that 28 (26%) of these 107 leptospirosis cases were dual infections is of concern especially since this diagnosis was made on serology tests only. The fact that blood cultures were not performed and only a minority were tested by PCR tests for typhoid and by serology tests for rickettsioses will mean that the frequency of these infections was under-estimated. Overall, this was a prospective study that was run for >1 year with well-defined recruitment criteria and a mostly standardised approach to assessing patients. It tested for most of the relevant infections in this area (but did not include blood cultures) and included exact definitions for both laboratory and clinical diagnoses. It also correlated the diagnoses made with the demographic, clinical and laboratory data collected and identified independent predictors of diagnoses, although the results were disappointing in this respect.

Another high-quality fever study in Thailand was performed in 5 different hospitals across the country from 2001-2 [Suttinont *et al.*, 2006]. The focus of the study was acute undifferentiated febrile illnesses and the recruitment criteria were less precisely defined according to the statement that, *"Most adult patients who … presented at one*

of these hospitals with acute fever (of <15 days' duration) without an obvious focus of infection were included in the study." Thereafter a standardised approach was followed and all patients had routine haematology, biochemistry, renal and liver function tests performed as well as urinalysis and chest radiography. Blood was sent for leptospiral cultures (but only occasionally for bacterial cultures) and also for acute and convalescent serology tests using samples from the admission and 2-4 weeks after discharge. 577 (68%) of 845 patients recruited had confirmed laboratory diagnoses as shown in Table 1.3.4.3, which is a simplified version showing details of dual infections. Although diagnoses were correlated with geographical location and seasonality, no correlations were made with other demographic, clinical or laboratory features.

Table 1.3.4.3. Diagnosis of undifferentiated febrile illnesses in Thailand (2001-2)

Diagnosis	**Cases***	**Percentage**
Leptospirosis	312	37%
Scrub typhus	135	16%
Dengue	44	5%
Influenza	39	5%
Murine typhus	15	2%
Rickettsia helvetica infection	8	1%
Q fever	7	1%
Japanese encephalitis	3	< 1%
Melioidosis	3	< 1%
Other bacterial infections	7	1%
Unknown	268	32%

* N = 845

Overall, this was a prospective study that was run for 1 year with poorly-defined recruitment criteria and a mostly standardised approach to assessing patients. It tested for most of the relevant infections in this area (but did not include bacterial

blood cultures) and it did not include clinical diagnoses. It also did not correlate the diagnoses made with the demographic, clinical and laboratory data collected.

1.3.5 Vietnam

No comprehensive fever studies have been performed in Vietnam, but the following detailed case definition was used in a study to evaluate some novel serology tests for leptospirosis [Wagenaar *et al.*, 2004] :

"To be considered a febrile patient, an individual had to present with an axillary temperature of >38°C and to have been febrile for <2 weeks. Patients found smear-positive for malaria (by microscopy), those with severe disease requiring referral (such as meningitis or sepsis), or signs and symptoms indicating a specific localised disease (such as tonsillitis, pneumonia, abscesses, peritonitis, arthritis, pyelonephritis) or chronic underlying conditions such as renal or hepatic disease, diabetes mellitus, HIV infection, and those taking immunosuppressive medication were excluded."

Although not stated as such, this is clearly an attempt to define patients with undifferentiated febrile illnesses. Similarly, in a study of brucellosis seroprevalence in Vietnam, the following definition was used for such illnesses [Nga *et al.*, 2006] :

"Acute undifferentiated fever was defined as any febrile illness of duration less than 14 days, confirmed by an axillary temperature ≥38.0 °C, without any indication for either severe systemic or organ specific disease."

Both of these studies are designed to investigate a single disease and so have little to add to the previous studies with regard to designing a comprehensive fever study in Sri Lanka. However, the attempts to define undifferentiated febrile illnesses are of interest since there is considerable variation in this respect amongst all of the previous studies.

1.3.6 Statistical Analysis

Most fever studies will perform a simple descriptive analysis of the diagnoses made and comment on how representative these findings are likely to be for the population in that area on a seasonal basis. Ideally, a fever study should also collect demographic, clinical and laboratory data that can be correlated with the diagnoses made. In two of the studies reviewed [Murdoch *et al.*, 2004; Ellis *et al.*, 2006] this was done using univariate analysis and then multivariate logistic regression. The data for each diagnosis were compared to the remainder of the cohort using appropriate univariate tests for dichotomous, ordinal and continuous variables. Those variables associated with a diagnosis at a significance of $P < 0.1$ then underwent multivariate logistic regression and those with a significance of $P < 0.05$ after regression were considered to be independent predictors of that diagnosis. Unfortunately, in both of these studies the results of this process did not identify many clinically useful predictors for specific infections (Tables 1.3.3.1 & 1.3.4.2) and therefore it was not possible to construct clinical prediction rules to help in diagnosing these diseases.

Most prediction rules that relate to infectious diseases are aimed at identifying a mortality risk [Lim *et al.*, 2003] or a clinical syndrome such as community-acquired pneumonia [Hopstaken *et al.*, 2003; Flanders *et al.*, 2004; Falguera *et al.*, 2009; Bilkis *et al.*, 2010], bacteraemia [Bleeker *et al.*, 2007; Shapiro *et al.*, 2008] or urinary tract infection [Gorelick *et al.*, 2003]. Rules that aim to identify specific infectious diseases are less common and mostly relate to distinguishing between different forms of meningitis [Patel *et al.*, 2010; Cohn *et al.*, 2012; Hristea *et al.*, 2012].

So far there are published rules for only one of the infections that present with an acute undifferentiated febrile illness in Sri Lanka. These were for enteric fever of which one was from Malaysia, but was not validated [Ross & Abraham, 1985] and the other was from Turkey [Hosoglu *et al.*, 2006], but the range of infections causing acute undifferentiated febrile illnesses was much smaller than in Sri Lanka. This suggests that developing a clinical prediction rule to distinguish between different causes of acute undifferentiated febrile illness in Sri Lanka will be difficult. However, it remains a worthwhile aim since it could avoid the unnecessary use of valuable or scarce resources – including investigations, antibiotics and hospital beds.

1.3.7 Lessons Learnt from Previous Studies

Previous fever studies in this region are usually focussed on :

- Identifying bacteraemias (blood stream infections) using blood cultures
- Identifying other infections that require serology or PCR tests
- Correlating diagnoses to demographic, clinical and laboratory findings

However, none of the studies reviewed here perform well all in three of these areas and none of the published papers indicate how representative the study population was in comparison to eligible patients who were not recruited for various reasons. This was often due to a lack of systematic recruitment and no data on recruitment rates in different groups was published in any of these studies. For example, in some countries a woman may feel that they can not join a study without their husband's permission or may feel less able to return for follow-up.

The ideal fever study in south or south-east Asia should :

- Be prospective
- Run continuously for ≥1 year
- Precisely define the patients to be recruited
- Obtain informed written consent from participants
- Keep demographic details of eligible patients who were not recruited
- Record demographic, clinical & laboratory data in a standardised format
- Include malaria tests, blood cultures & paired serology tests for relevant pathogens
- Use precise definitions for confirmed/laboratory & unconfirmed/clinical diagnoses
- Correlate the final diagnoses with the demographic, clinical & laboratory data
- Seek to develop clinical prediction rules to help in diagnosing the infections found
- Assist in the development of appropriate diagnostic tests for use in the host country

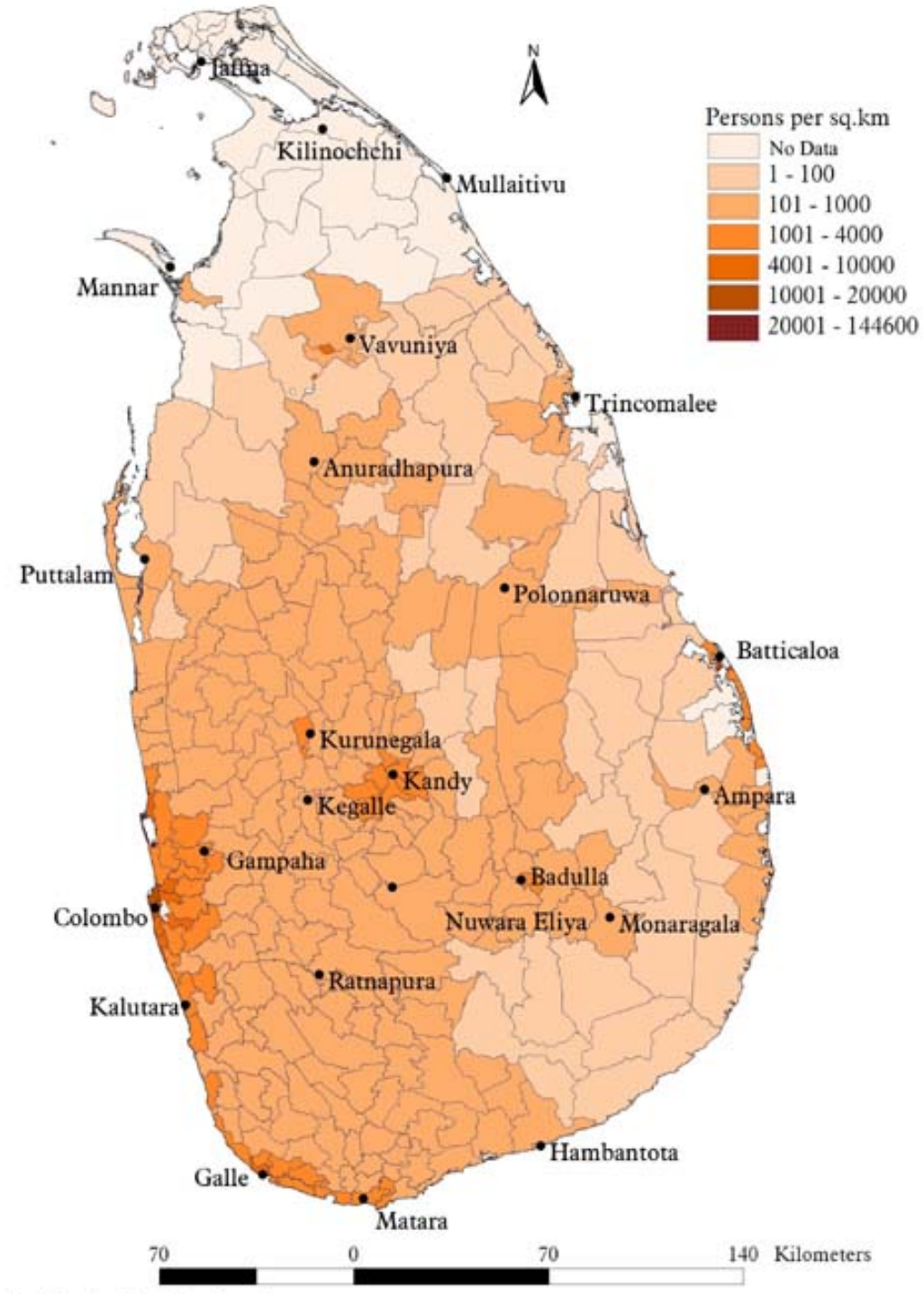
1.4 Situation and Objectives

Some variation in the way that fever studies are performed is due to the situation in which they are conducted and these local factors must be considered when designing a study such as this. Fever studies are usually hospital-based to ensure that adequate numbers of patients with the most serious infections are recruited, but this does mean that their findings are not necessarily representative of the wider population.

1.4.1 Geographical Situation

Sri Lanka is an island measuring 270 by 140 miles that is located within the tropics and lies 22 miles from the south-east coast of India. In keeping with its tropical location it lacks summer and winter seasons, but has rainy seasons instead. The south-west part of the island is called the “wet zone” and has two rainy seasons per year, whereas the north-eastern part is called the “dry zone” and has only a single rainy season per year. The total population is ~20 million and this is distributed as shown in Figure 1.4.1. Sri Lanka was previously part of the British Empire and called Ceylon, but changed its name at independence in 1948. The country’s economic development has been hindered by a succession of civil conflicts (including the Tamil conflict from 1983-2009) and natural disasters (including the Indian Ocean tsunami in 2004), which mean that it remains a developing country.

Figure 1.4.1. Population density of Sri Lanka in 2001 (© 2012 recoverlanka.net)



1.4.2 Medical Situation

Medical facilities in Sri Lanka include a network of hospitals of various sizes that extend across the country according to the population distribution. Laboratory facilities can be very limited in the smaller hospitals and only the six large teaching hospitals (Colombo, Colombo North, Colombo South, Kandy, Galle and Jaffna) had dedicated medical microbiologists at the time of this study. Hence these were the best places to conduct a fever study and Colombo North Teaching Hospital (CNTH) at Ragama in the Gampaha District of western Sri Lanka was especially appropriate since it serves a large population of ~2 million people that live in both urban and rural settings. Its location is shown in Figures 1.4.2.1 and 1.4.2.2.

Figure 1.4.2.1. Location of CNTH at Ragama in Sri Lanka (© 2012 Google)

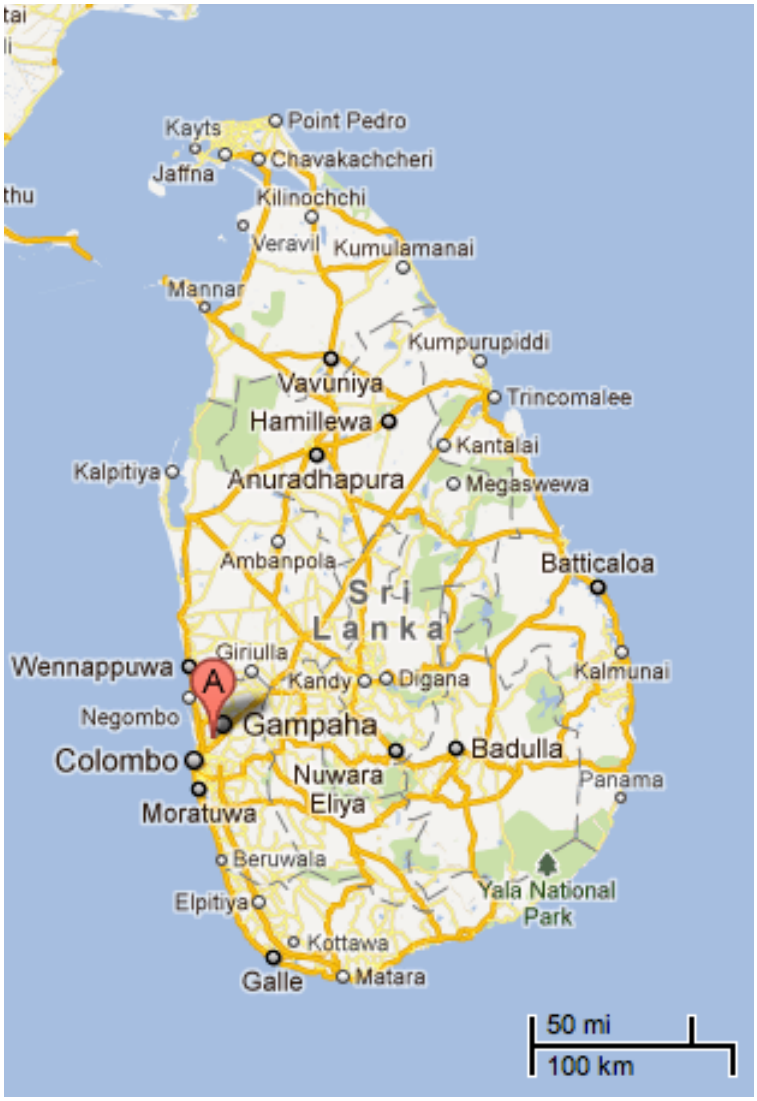
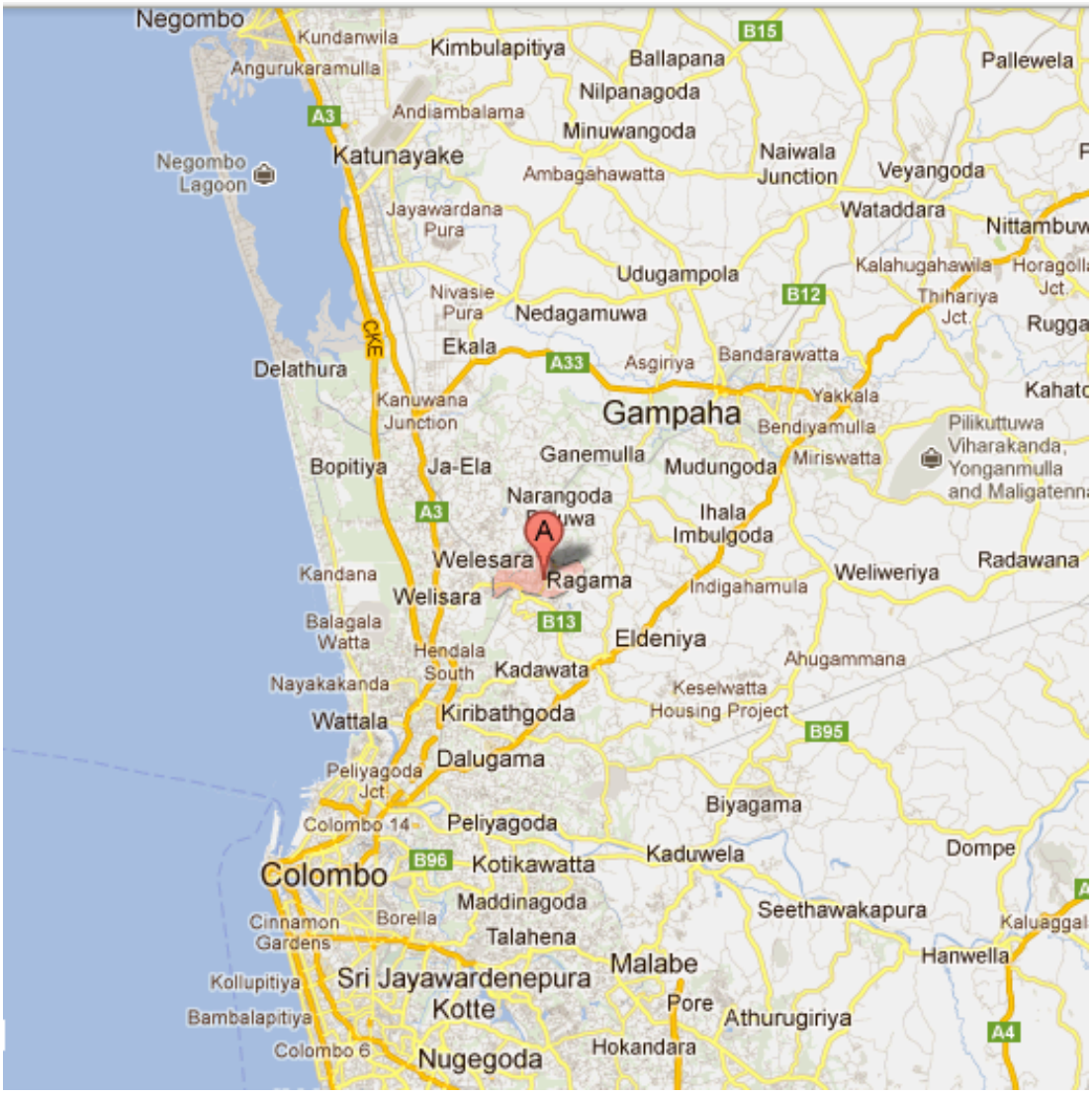


Figure 1.4.2.2. Location of Ragama in Relation to Colombo and Katunayake (© 2012 Google)



CNTH is a large general hospital and is co-located with the Faculty of Medicine of the University of Kelaniya. The hospital has 8 adult general medicine wards and 2 of these (one for males and one for females) form the Professorial Medical Unit, which is staffed by academic consultants and offers a good setting for clinical research projects. CNTH has its own pathology laboratories that do basic haematology, biochemistry and microbiology investigations, but the Department of Microbiology at the Faculty of Medicine has better facilities for research. Even so at the time this study began there was no routine blood culture facility available within this department. Private laboratories in Colombo are able to do additional investigations

Running a microbiology laboratory remains a laborious business requiring more skilled staff and a wider range of supplies in comparison to modern haematology, biochemistry and even molecular diagnostic laboratories. Therefore microbiology services in developing countries are often limited and clinicians must become accustomed to treating suspected infection cases with empirical antibiotics. However, the widespread over-use of anti-microbial drugs and inability to report local microbial sensitivities means that there is a significant risk of antibiotic resistance and treatment failures.

Many of the infections that occur in Sri Lanka can not be identified using conventional parasitology and bacteriology techniques and the necessary specialist cultures, serology and PCR tests would be difficult to introduce and maintain on a regular basis. Therefore a fever study that was able to develop clinical prediction rules for these infections would be most valuable. Rules with a high sensitivity would enable efficient use of laboratory diagnostics to confirm infections, whereas rules with a high specificity would enable the efficient use of hospital beds for admitting patients with life-threatening infections and discharging those who did not. The latter is especially important during the rainy seasons when there are insufficient hospital beds for the increased number of febrile illness patients that present.

1.4.3 Objectives

In accordance with the aims of the study (Section 1.1.1) the following objectives were established :

1. To design a fever study that was appropriate to the diseases, facilities and needs of a hospital serving a mixed urban and rural population in Sri Lanka.

2. To submit this research proposal for approval of its scientific merit, research ethics and estimated costs to the University of Kelaniya, Liverpool School of Tropical Medicine and Defence Postgraduate Military Deanery.

3. To recruit and train an appropriate number of medical research assistants to help with the recruitment and assessment of patients (especially out of hours) thus helping to ensure that the study sample was as complete and representative as possible.

4. To establish a safe and reliable system for transporting diagnostic samples in optimum conditions to a range of international reference laboratories.

5. To create an electronic database to store anonymous patient data – including demographics, clinical features, laboratory results and final diagnoses obtained from the reference laboratories.

6. To conduct the study over a 1-year period with minimal interruption and regular progress meetings to minimise problems.

7. To analyse the diagnostic results and correlate these to the demographic, clinical and laboratory data collected and if possible, create clinical prediction rules for the most common infections found.

8. To use an appropriate rapid diagnostic test prospectively during the study in order to assist in its evaluation and then provide data and samples for retrospective use in the evaluation of other such tests.

Chapter 2

Methods

2.1 Patient Recruitment, Data Collection and Sample Collection

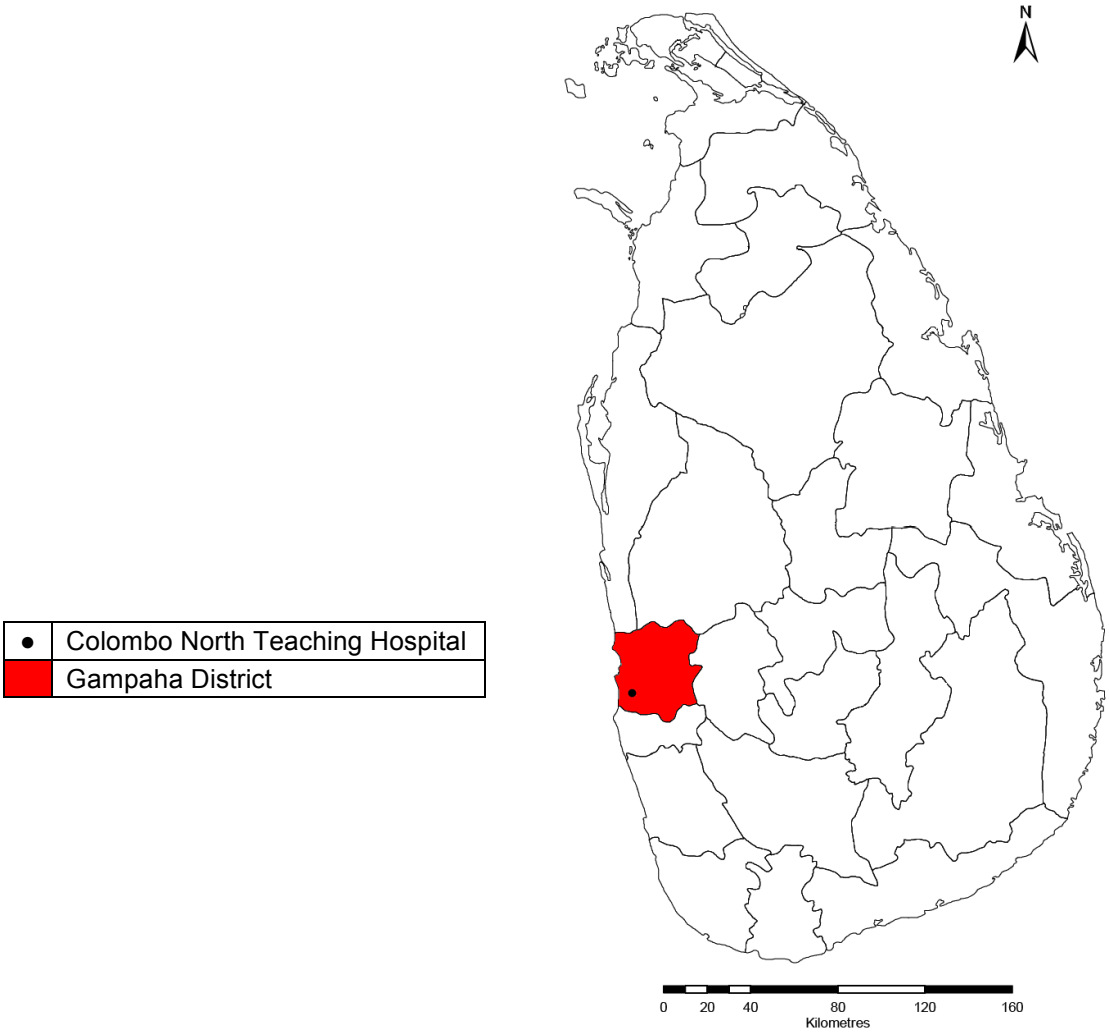
2.1.1 Patient Recruitment

This was a prospective and systematic study carried out at the Colombo North Teaching Hospital (CNTH) at Ragama in the Gampaha District of western Sri Lanka (Figure 2.1.1) over a one-year period from 06 June 2006 to 05 June 2007. This hospital serves a population of ~2 million people from both urban and rural areas and patients were recruited from the male and female wards of the Professorial Medical Unit (PMU), which is responsible for 25% of all adult medical admissions on an unselected basis.

Patients with an oral temperature ≥38.0 °C on admission were eligible to join the study, but were excluded if they did not provide informed written consent, were aged <16 years, had been admitted for >24 hours or if they had already received antibiotics in hospital. Patients were recruited using the patient information and consent form shown in Appendix 1.

One key objective was to ensure that the study sample was as complete and representative as possible and so three medically-qualified research assistants were employed to assist with the recruitment and assessment of patients. They received specific training and daily supervision for all aspects of their work and visited every new admission on the PMU wards to assess their eligibility for the study. Between them they were able to provide 24-hour cover of the wards in order to avoid problems with out-of-hours recruitment. Without this cover eligible patients would have been missed and become ineligible by exceeding the 24-hour deadline or by receiving antibiotics in hospital before they could be recruited. The total number of patients admitted to the PMU wards and the demographic details of patients not recruited were also collected and reviewed monthly to detect any bias in recruitment.

Figure 2.1.1. Location of CNTH and the Gampaha District within Sri Lanka



2.1.2 Data Collection

Recruited patients had demographic, epidemiological, clinical and laboratory data recorded by the research assistants using the data collection form shown in Appendix 2. This form was updated 2 months into the study when a patient presented with imported malaria to include a question on travel overseas. Additional questions regarding the clinical features of enteric fever were also added once cases were identified. Otherwise the data collection remained the same throughout the study. Recruited patients were visited every day by one of the study team to update the data collection form with any new clinical or laboratory features. They were all requested to attend the out-patients clinic on a single occasion 2-4 weeks after discharge for clinical review and further data collection and were offered LKR 200 (~£1) to cover

travelling expenses if they did attend. Those that did not attend were sent a single reminder card by post.

In order to understand any seasonal variation in admissions, monthly rainfall data for the Gampaha District was obtained from the (National) Department of Meteorology, Sri Lanka. These measurements were made at the Katunayake Airport weather station, which is located 15 km north of CNTH as shown in Figure 1.4.2.2.

In order to describe the distribution of cases in Gampaha District, electronic maps for the ArcGIS mapping software package were obtained from the Department of Geography, University of Kelaniya, Sri Lanka. These maps were sufficiently detailed to identify each Grama Niladara (GN) division, which is the smallest administrative division used in Sri Lanka and is similar to a UK post code.

In order to identify any variation in the distribution of cases between urban and rural areas, population data for each GN division in Gampaha District was obtained using the 2001 Census from the (National) Department of Census and Statistics, Sri Lanka. For each GN division the area from the electronic maps and the population from the census records were combined in a spreadsheet to calculate the population density. Since there is no definition of urban or rural areas in Sri Lanka, the definitions used in India and certain other countries were used instead [Government of India, 2011; Statistics Canada, 2011]. Hence an urban area was defined as having ≥400 people / km^2 and a rural area was defined as having <400 people / km^2.

In order to compare the diagnoses made in this study with other public health records, the weekly notifiable disease records for Gampaha and other Districts from 2006-7 were obtained from the (National) Epidemiology Unit, Sri Lanka. However, it should be noted that these cases are usually diagnosed on clinical criteria only and the data included in these reports are not considered very reliable.

All data were entered into a FileMaker Pro database by myself and 5% of all data entry was independently validated. All computer files were encrypted and stored securely with a back-up performed each week using both a password-protected external hard disk and a secure Internet storage system (www.mozy.com).

2.1.3 Sample Collection

Diagnostic samples were taken by the research assistants with help from me whenever required. On the day of recruitment 20 ml of blood was obtained by aseptic technique from each patient and divided equally amongst:

- an in-house blood culture bottle for bacteria
- a BacT/ALERT blood culture bottle for bacteria
- a heparinised culture bottle for leptospires
- a plain tube for CRP and CPK tests

These samples were kept at room temperature (25-30 °C) for up to 4 hours before being delivered to the laboratory. Culture bottles for bacteria were incubated at 37 °C, whereas those for leptospires were kept at room temperature (25-30 °C) for up to 2 weeks before being sent overseas using Aramex's medical specimen service. Also on the day of recruitment a urine sample was collected for assays to measure protein content and detect the presence of antimicrobials. These urine samples were stored in a fridge at -4 °C for up to 4 hours before being delivered to the laboratory where they were stored at -30 °C until the time of testing. On the day following recruitment 20 ml of blood was taken and kept at room temperature (25-30 °C) for up to 4 hours before being centrifuged to produce serum that was divided equally between 5 microtubes. These serum samples were stored at -80 °C and transported with dry ice to laboratories overseas using DHL's Medical Express service. Recruited patients were also asked to give 20 ml of blood for repeat serology tests on discharge (if they had been admitted for >5 days) and on follow-up 2-4 weeks later.

2.2 Laboratory Investigations

The microbiology investigations used in this study were all gold standard tests performed at research and reference laboratories in several different countries. All other laboratory investigations were of the highest standard available in Sri Lanka.

2.2.1 Malaria Investigations

Malaria was diagnosed by microscopy of thick blood films stained with Giemsa at the Department of Parasitology, University of Kelaniya, Sri Lanka.

2.2.2 Bacteraemia Investigations

Bacteraemia was diagnosed using two different aerobic blood culture bottles for each patient at the Department of Microbiology, University of Kelaniya, Sri Lanka. Ten ml of blood was collected using aseptic technique and divided equally between an in-house culture bottle and a BioMérieux BacT/ALERT FA culture bottle (Catalogue no. 259791, Batch no. 1014621; BioMérieux, Durham, NC). The in-house bottles contained a standard brain heart infusion (BHI) medium with 0.05% sodium polyanethol sulfonate (SPS) as recommended by the Sri Lanka College of Microbiologists [Thevanesam, 2001]. The BacT/ALERT bottles contained a commercial peptone-enriched tryptic soy broth supplemented with BHI and also activated charcoal that was supposed to neutralise any antimicrobials already taken by the patient.

Both bottles were incubated at 37 °C and sub-cultured at 1, 2 and 7 days onto blood, chocolate and MacConkey agar plates. Sub-culturing of the in-house bottles was done by removing the lid and inserting a loop, whereas the BacT/ALERT bottles had a sub/venting needle left in place. All sub-culture plates were incubated at 37 °C for 48 h with blood and chocolate plates in 5-10% CO_2 and MacConkey plates in air. Identification of bacteria and their antibiotic sensitivities was done in Sri Lanka using standard laboratory techniques that included the use of the commercial IDS Rapid microbial identification panels (Span Diagnostics Ltd, India) and the Kirby-Bauer antibiotic disk diffusion technique. These isolates were later transferred in concentrated BHI broth with dry ice to the Department of Medical Microbiology, University of Liverpool, UK where their identification and antibiotic sensitivities were confirmed using similar techniques and where necessary by 16S rRNA gene sequence analysis to confirm identities.

The in-house bottles were made of glass and were reused after cleaning and autoclaving, whereas the BacT/ALERT bottles were made of plastic and were disposed of after autoclaving.

2.2.3 Other Bacterial Investigations

Other bacterial infections were diagnosed from urine and sputum samples using standard microbiology techniques at the Department of Microbiology, University of Kelaniya, Sri Lanka. These specimens were collected as part of the patients' routine management by clinicians on the Professorial Medical Unit and not in a systematic way relating to the study. Urine cultures were done quantitatively using a calibrated 1 µl wire loop and cysteine lysine electrolyte-deficient (CLED) agar plates, which were incubated at 37 °C in room air for 48 h. Sputum cultures were performed on samples that passed Murray-Washington quality criteria [Murray & Washington, 1975] using blood and chocolate agar plates, which were incubated at 37 °C in 5-10% CO_2 for 48 h.

2.2.4 Tuberculosis Investigations

Tuberculosis (TB) was diagnosed in conjunction with the (National) Chest Hospital at Welisara, which is 5 km away from Ragama and the only specialist TB hospital in Sri Lanka. TB cultures are not performed on all cases due to a relative lack of resources in comparison to the number of patients seen. Hence "clinically-typical" pulmonary TB cases will usually not have cultures done, but will be started on anti-tuberculous therapy and followed up to ensure there is a good clinical response. For the purpose of this study a "clinically-typical" case of pulmonary TB was defined as having all of the following:

- suggestive clinical features *eg.* fever, cough, sputum, dyspnoea, weight loss
- a positive Mantoux test (>10 mm with 1 unit of purified protein derivative)
- raised inflammatory markers (ESR ≥40 mm/h or CRP ≥48 mg/L)
- pulmonary cavitation or pleural effusion on chest radiography
- no alternative respiratory diagnosis

(The levels chosen to define raised inflammatory markers were expected to occur in bacterial infections such as TB and the CRP figure reflects the serial dilutions used to measure this as described in Section 2.2.15) Hence in this study TB cases were defined as laboratory-confirmed TB (on culture or histology) or as "clinically-confirmed" pulmonary TB (with a documented response to anti-tuberculous therapy).

2.2.5 Brucellosis Investigations

Brucellosis was diagnosed from serum samples using an established protocol of tests at the (National) Brucellosis Reference Unit, UK as described previously [Sharma *et al.*, 2008]. Initially, serum samples were screened at a dilution of 1:160 with the commercial Brucellacapt immunocapture-agglutination assay (Vircell SL, Granada, Spain), which has performed well in several independent evaluations [Orduña *et al.*, 2000; Casao *et al.*, 2004; Gómez *et al.*, 2008].

The Brucellacapt assay was performed using 50 µl samples of 1:160 diluted sera, which were added to the wells of a U-bottom microtitre plate coated with anti-total human immunoglobulin. Then 50 µl of an antigen suspension (coloured *Brucella melitensis* bacteria killed by formaldehyde treatment) was added to each well and plates were incubated at 37 °C for 24 h. Positive reactions showed agglutination spread over the bottom of the well, whereas negative reactions were indicated by a pellet at the centre of the well.

Serum samples that were Brucellacapt positive at a dilution of 1:160 were then further investigated using a series of validated in-house tests [Sharma *et al.*, 2008]. Enzyme immunoassays (EIAs) for IgM and IgG antibodies were performed and if positive these were followed by a micro-agglutination (MAG) assay and a complement fixation test (CFT) as described previously [Sharma *et al.*, 2008].

The EIAs were performed using whole cell *Brucella abortus* antigen strain S99 obtained from the Veterinary Laboratory Agency (VLA), Weybridge, UK along with horseradish peroxide labelled conjugate (anti-human IgM or IgG depending on which assay was being performed) and enzyme substrate in a standard EIA format. Test sera were added using a dilution range of 1:20 to 1:10240 in a 96-well EIA

plate, which was incubated at 37 °C for 45 min and then read using a spectrophotometer at 450/620 nm within one hour of stopping the reaction. A negative reaction was indicated by an optical density (OD) ≤0.8 and a positive reaction was indicated by an OD >0.8. The end-point titre of the test serum was derived from the highest dilution with an OD >0.8. A negative result was defined as a titre of <1:20 and positive results were defined as those with titres of ≥1:20.

The MAG assay was performed using safranin-stained whole cell *B. abortus* antigen strain S99 from VLA, UK. Test sera were added using a dilution range of 1:20 to 1:2560 in a V-bottomed plate, which was incubated at 37 °C for 24 h and then read by naked eye. A negative reaction with no agglutination was indicated by a button of red-stained bacteria in the centre of the well with a surrounding pink halo. However, if *Brucella* antibodies (IgG, IgM or IgA) were present, then an antigen-antibody complex was formed leading to agglutination, which resulted in a mat of stained cells spread over the bottom of the well or a diminished button of bacterial cells in the centre of the well with a slightly opaque surrounding diluent. The end-point titre of the test serum was derived from the highest dilution that showed agglutination. A negative result was defined as a titre of <1:20 and positive results were defined as those with titres of ≥1:20. Titres of ≥1:160 were considered to be clinically significant, whereas titres of 1:20 to 1:160 were considered to be of indeterminate clinical significance.

The CFT was performed using whole cell *B. abortus* antigen strain S99 from VLA, UK along with complement and sensitised sheep erythrocytes in a standard CFT format. Test sera were added using a dilution range of 1:4 to 1:256 in a U-bottomed microtitre plate, which was incubated at 37 °C for 24 h and then read by naked eye. A negative reaction with no complement fixation was indicated by complete lysis of sensitised sheep erythrocytes (lysis score four). However, if *Brucella* antibodies (IgG, IgM or IgA) were present, then an antigen-antibody complex was formed leading to fixation of the complement and no lysis, which resulted in a button of erythrocytes at the centre of the well (lysis score zero). The end-point titre of the test serum was derived from the highest dilution that showed 50% or more fixation (lysis score two). A negative result was defined as complete lysis at 1:4 dilution and positive results were defined as those with titres of >1:4.

2.2.6 Leptospirosis Investigations

Leptospirosis was diagnosed by blood cultures at the Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand and from serum samples using a validated in-house IgM ELISA and the micro-agglutination test (MAT) at the (National) Leptospira Reference Unit, UK.

Blood cultures were performed using methods described previously [Wuthiekanun *et al.*, 2007]. Blood was collected into 5 ml polystyrene tubes (Teklab, UK) containing 25 U sodium heparin (Leo Pharma, UK) and kept at 25-30 °C throughout. Aliquots of whole blood and centrifuged plasma deposits were cultured in 0.1% semi-solid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with 3% normal rabbit serum at 25-30 °C for 13 weeks with weekly checks using dark-field microscopy. Isolates underwent multi-locus sequence typing (MLST) as described previously [Thaipadungpanit *et al.*, 2007] and were also sent to the WHO/FAO/OIE Collaborating Centre for Reference & Research on Leptospirosis, Brisbane, Australia for serovar identification using the cross-agglutinin absorption test (CAAT) [Stallman, 1984].

The IgM ELISA and MAT were performed using methods described previously [Zochowski *et al.*, 2001; Palmer *et al.*, 1987; Wolff, 1954]. The former was used to screen samples and positives were then confirmed using the latter in accordance with the usual reference laboratory protocol.

The ELISA was performed using heat-extracted antigen from a culture of *L. interrogans* serovar Hardjo strain Hardjo-Prajitno [Terpstra *et al.*, 1980]. Test sera were added using a dilution range of 1:20 to 1:2560 in a standard ELISA plate, which was incubated at 37 °C for 60 min prior to the addition of peroxidise conjugated anti-human IgM and the necessary chromogenic substrate. Plates were read using a spectrophotometer at 405 nm and a positive reaction was indicated by an OD >0.18. The end-point titre of the test serum was derived from the highest dilution with an OD >0.18. Negative results were defined as those with a titre of <1:80, equivocal results were defined as those with titres of 1:80 to 1:160 and positive results were defined as those with titres of ≥1:320.

The MAT was performed using a battery of leptospiral antigens from reference strains. These were combined into the following *Leptospira interrogans* serogroups by pooling appropriate serovars (shown in brackets): Australis (australis, bratislava); Autumnalis (bangkinang, bim, bulgarica); Ballum (ballum); Bataviae (bataviae, djatzi); Canicola (broomi, canicola); Celledoni (anhoa, whitcombi); Cynopteri (cynopteri); Grippotyphosa (grippotyphosa, valbuzzi); Hebdomadis (hebdomadis); Icterohaemorrhagiae (icterohaemorrhagiae); Javanica (fluminense, javanica); Mini (beye, georgia, perameles); Pomona (pomona, proechimys); Pyrogenes (pyrogenes, robinsoni); Sejroe (hardjo; saxkoebing; sejroe) Tarassovi (rama, tarassovi) and *L. biflexa* serogroup Semaranga serovar patoc was also included. Negative results were defined as those with a titre of <1:80, equivocal results were defined as those with titres of 1:80 to 1:160 and positive results were defined as those with titres of ≥1:320 or a fourfold rise in titre between admission and convalescent serum samples. The presumptive infecting serogroup was taken as that which gave the highest MAT titre with a convalescent serum.

2.2.7 Q fever Investigations

Q fever was diagnosed from serum samples by immunofluorescence assay (IFA) using phase I and phase II antigens from *C. burnetti* at the Unité des Rickettsies, Marseille, France as described previously [Dupont *et al*., 1994]. Acute Q fever was diagnosed if anti-phase II antibody titres were ≥200 for IgG and ≥50 for IgM or if seroconversion was demonstrated. Chronic Q fever was diagnosed if anti-phase I antibody titres for IgG were ≥800. Probable Q fever was diagnosed if anti-phase II antibody titres were <200 for IgG but ≥50 for IgM because these cases probably represent early acute infections. Evidence of past Q fever infection was diagnosed if anti-phase II antibody titres were ≥100 for IgG and undetectable for IgM.

2.2.8 Rickettsia Investigations

Rickettsial infections were diagnosed from serum samples by IFA using a panel of 13 rickettsial antigens, which included *Rickettsia prowazekii*, *R. typhi*, spotted fever group (SFG) rickettsiae (*R. japonica*, *R. helvetica*, *R. slovaca*, *R. conorii* subsp.

indica, *R. honeï*, *R. heilongjangensis*, and *R. felis*), and *O. tsutsugamushi* (Gilliam, Karp, Kato and Kawasaki strains) at the Unité des Rickettsies, Marseille, France as described previously [La Scola *et al*., 1997]. Confirmed rickettsial infection was diagnosed if antibody titres were ≥64 for IgG and ≥32 for IgM or if seroconversion was demonstrated. Positive results with cross-reacting antibodies to multiple *Rickettsia* species were resolved by western blotting with cross-adsorption using the antigens listed. Due to some unexpected negative results for patients with obvious eschars the samples were later re-tested for scrub typhus at the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, Thailand using the same antigens as above. Probable rickettsial infection was diagnosed in patients with eschars and good responses to doxycycline, who did not meet the criteria for a confirmed infection.

2.2.9 Dengue Investigations

Dengue was diagnosed from serum samples by IgM and IgG capture ELISAs and nested RT-PCR tests at the Armed Forces Medical Research Institute for Medical Sciences (AFRIMS), Bangkok, Thailand and by virus isolation at the (National) Novel and Dangerous Pathogens (NaDP) Unit, Porton Down, UK.

The ELISAs were performed and interpreted as described previously [Innis *et al.*, 1989; Vaughn *et al.*, 1999; Blacksell *et al.*, 2012]. Antigens for dengue virus types 1 to 4 (DENV-1 to DENV-4) were produced by sucrose acetone extraction of the brains of suckling mice infected with the following prototype mouse adapted virus strains: DENV-1 Hawaii, DENV-2 New Guinea C, DENV-3 H-87 and DENV-4 H-241. Acute dengue infection was diagnosed if the admission sample had ≥40 U of dengue IgM antibodies (with dengue IgM ≥ JEV IgM antibodies) or if paired samples had <15 U of dengue virus IgM antibodies in the admission sample increasing to ≥30 U in the convalescent sample (with dengue IgM ≥ JEV IgM antibodies). Primary and secondary infections were determined when the ratio of anti-dengue virus IgM to IgG was ≥1.8 or <1.8 respectively. In the absence of diagnostic IgM levels, a 2-fold increase in IgG to a value ≥100 U was also considered indicative of acute secondary dengue infection. Recent (non-acute) dengue infection was diagnosed if dengue IgM antibodies were below diagnostic

levels and IgG antibodies were >100 U in the acute sample, but failed to rise in the convalescent sample.

The same diagnostic approach using the AFRIMS dengue (DEN) IgM antibody capture (MAC) ELISA, IgG antibody capture (GAC) ELISA and equivalent Japanese Encephalitis Virus (JEV) MAC and GAC ELISAs for serological diagnosis has been described in various ways. The most concise and recent version of this is shown below and summarised in Figure 2.2.9 [Pan-Ngum *et al*, 2012].

Acute primary dengue infections were defined if :

1. The admission sample showed DEN MAC ELISA ≥40 U, a ratio of DEN MAC ELISA to JEV MAC ELISA ≥1 and a ratio of DEN MAC ELISA to DEN GAC ELISA ≥1.8:1.

or

2. The admission sample showed DEN MAC ELISA in admission sample <15 U and in convalescent-phase specimen ≥30 U.

Acute secondary dengue infections were defined if :

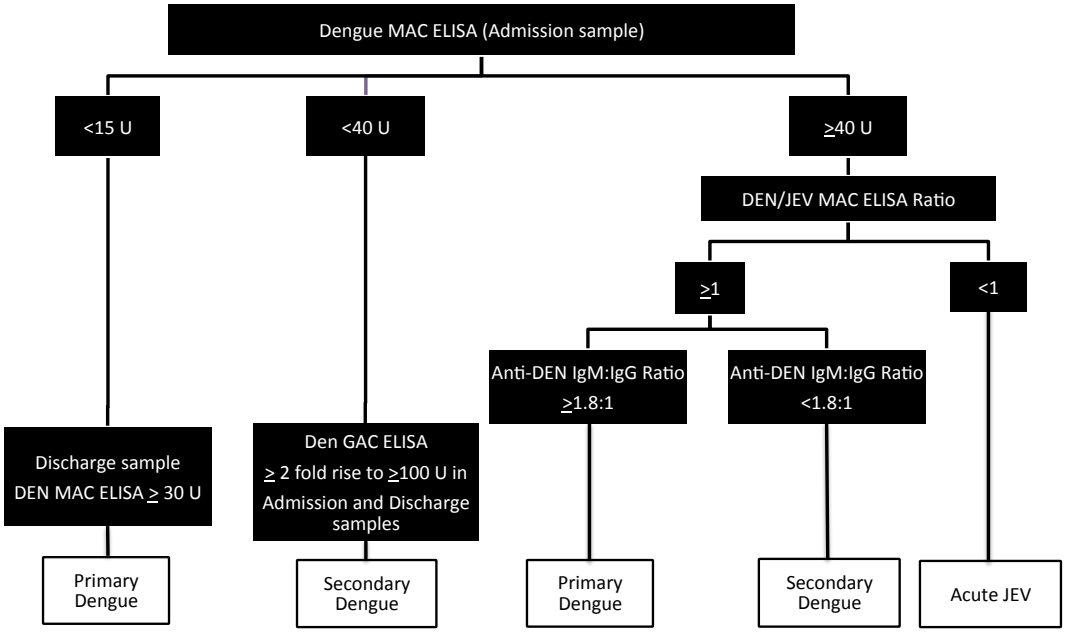
1. The admission sample showed DEN MAC ELISA ≥40 U, a ratio of DEN MAC ELISA to JEV MAC ELISA ≥1 and a ratio of DEN MAC ELISA to DEN GAC ELISA <1.8:1.

or

2. The admission sample showed DEN MAC ELISA <40 U, a ratio of DEN GAC ELISA in convalescent-phase specimen to admission specimen ≥2 (2-fold rise) and DEN GAC ELISA in convalescent-phase specimen ≥100.

(Acute JEV infection was defined if the admission sample showed DEN MAC ELISA ≥40 U and a ratio of DEN MAC ELISA to JEV MAC ELISA <1.)

Figure 2.2.9. Flow chart describing the AFRIMS dengue diagnostic algorithm



The RT-PCR tests were performed and interpreted as described previously [Lanciotti *et al.*, 1992], but were modified by the use of avian myeloblastosis virus reverse transcriptase, a 12.5-pmol primer concentration and a nested RT-PCR amplification of 25 cycles. This RT-PCR uses nested primers to produce amplicons of different sizes for each dengue virus type and so a type-specific result was obtained. RT-PCR tests were done on all admission samples except for cases that were proven to be dengue serology negative on paired tests.

Dengue virus isolations were performed on admission samples that were RT-PCR positive using Vero E6 cells (ATCC CRL1586) grown in modified Eagle's medium at 37 °C using Bio-Safety Level 3 (BSL-3) facilities as described previously [Ter Meulen *et al.*, 2000]. Cultures were inspected daily and any that showed a cytopathic effect (CPE) had supernatant analysed by RT-PCR (as above) to confirm the presence of dengue virus.

2.2.10 Japanese Encephalitis Investigations

Japanese encephalitis virus (JEV) infections were diagnosed from serum samples by IgM and IgG capture ELISAs at AFRIMS, Bangkok, Thailand as described previously [Burke & Nisalak, 1982; Innis *et al.*, 1989]. Antigens for JEV were produced by sucrose acetone extraction of the brains of suckling mice infected with the prototype mouse adapted virus strain JEV Nakayama. Acute JEV infection was diagnosed if the admission sample had ≥40 U of JEV IgM antibodies (with JEV IgM > dengue IgM antibodies) as described previously in Section 2.2.9.

2.2.11 Chikungunya Investigations

Chikungunya was diagnosed from serum samples by haemagglutination inhibition (HAI) and enzymed immunosorbent assays (EIA) for chikungunya virus IgM antibodies at AFRIMS, Bangkok, Thailand and by a real-time RT-PCR test and virus isolation at the NaDP Unit, Porton Down, UK.

The HAI assays were performed and interpreted as previously described [Clarke & Casals, 1958]. Antigens for chikungunya virus (CHIKV) were produced by sucrose acetone extraction of the brains of suckling mice infected with a mouse adapted virus strain. The EIAs were performed using this same antigen and the same method as the AFRIMS dengue serology tests [Innis *et al.*, 1989]. Acute chikungunya infection was diagnosed if paired samples showed a 4-fold increase in titres on the HAI assay or if single samples had >40 U of chikungunya IgM antibodies on the EIA. However, testing of single samples was not able to determine if a chikungunya infection was acute or recent (non-acute) and so these cases must be considered indeterminate. Recent (non-acute) chikungunya infection was diagnosed if paired samples failed to show a 4-fold increase in titres on the HAI assay.

The real-time RT-PCR tests were performed as described previously [Edwards *et al.*, 2007] and were done on all samples including discharge and follow-up samples. During this study the RT-PCR appeared over-sensitive in comparison to the AFRIMS serology results for paired samples. This led to several inconsistent results in which samples were RT-PCR positive, but serology negative despite testing paired

samples. However, the RT-PCR results in question were all weakly positive and so a simple adjustment of the real-time RT-PCR crossing point was made to reduce the sensitivity of the test and so convert these apparent false positives into negatives. Doing so also removed some other cases that were weakly positive on RT-PCR and had provided admission serum samples only.

A selection of RT-PCR positive cases were further analysed with another RT-PCR test to amplify part of the chikungunya virus E1 gene [Edwards *et al.*, 2007]. These sequences were then used to determine the phylogenetic relationship between chikungunya viruses in this study and a variety of reference strains.

Chikungunya virus isolations were performed on admission samples that were RT-PCR positive using Vero E6 cells (ATCC CRL1586) grown in modified Eagle's medium at 37 °C using Bio-Safety Level 3 (BSL-3) facilities as described previously [Ter Meulen *et al.*, 2000]. Cultures were inspected daily and any that showed a cytopathic effect (CPE) had supernatant analysed by RT-PCR (as above) to confirm the presence of chikungunya virus.

2.2.12 Hantavirus Investigations

Hantavirus infections were diagnosed from paired serum samples only by commercial serology tests, a diagnostic RT-PCR in-house test and RT-PCR amplification plus sequencing of the small (S), medium (M) and large (L) segments of the hantavirus genome at the NaDP Unit, HPA Porton Down, UK.

Initially, serum samples were screened with the commercial Hantavirus IgG DxSelect ELISA (Catalogue no. EL1600G; Focus Diagnostics, Cypress, CA, USA), which is an ISO-certified test that has performed well in previous Asian studies [Chandy *et al.*, 2008]. This ELISA used a cocktail of hantavirus antigens from Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), Dobrava-Belgrade virus (DOBV) and Sin Nombre virus (SNV), which were coated onto polystyrene microwells and used to detect IgG antibodies against these virus types. The screening ELISA is an indirect test in which the optical density (OD) is

proportional to the antigen-specific IgG antibodies present in the sample. The results were obtained by comparison of the sample OD readings with reference cut-offs.

Serum samples that were hantavirus IgG positive were then further investigated using the commercial Hantavirus IgM DxSelect ELISA (Catalogue no. EL1600M; Focus Diagnostics, Cypress, CA, USA), which is the IgM version of the IgG ELISA used for screening samples. (This IgM ELISA produces too many false positives for it to be used as a screening test [J Osborne, personal communication].) All hantavirus IgG positive cases were also tested with commercial combined IgM/IgG IFAs for the HTNV, SEOV and PUUV types (Catalogue nos. PR77056, PR77060, PR77065, Progen, Heidelberg, Germany), which have performed well in previous studies and are considered the best available serology test overall [Koraka *et al.*, 2000]. These IFA tests were performed according to the manufacturer's instructions and a positive result was indicated by bright punctuate granular staining in the cytoplasm of the infected cells. The tests were repeated with serial dilutions of the sera in order to identify those that had a four-fold increase in titres, which was considered indicative of an acute infection.

Any cases with serological evidence of acute hantavirus infection underwent RT-PCR testing [Kramski *et al.*, 2007], which was designed to detect the major pathogenic hantaviruses found in Asia, Europe and America (HTNV, SEOV, PUUV, DOBV, TULV, SNV & ANDV). However, no positives were identified and so a series of degenerate primers for the hantavirus S segment [Reynes *et al.*, 2003], M segment [Nichol *et al.*, 1993] and L segment [Klempa *et al.*, 2006] were used to identify any possible hantavirus in these samples. Those with sequences suggestive of THAIV were re-tested using specific primers for that hantavirus [Pattamadilok *et al.*, 2006; Hugot *et al.*, 2006] in order to obtain more detailed sequence information. The sequences identified were compared with those for the hantavirus isolate obtained previously from rats in Sri Lanka [Vitarana *et al.*, 1988], which is now stored as strain "Sri R 87-1315" at the Korea University [JW Song, personal communication].

2.2.13 Other Virology Investigations

Other virus infections such as mumps and chickenpox were diagnosed clinically and confirmed by the virology investigations used at the appropriate reference laboratories of the Health Protection Agency (HPA), UK.

2.2.14 Urinary Antimicrobial Investigations

Antimicrobial activity in urine samples was identified by microbiological assays described previously [Liu *et al.*, 1999]. Reference isolates of *Bacillus stearothermophilus* (ATCC 7953), *Escherichia coli* (ATCC 25922) and *Streptococcus pyogenes* (ATCC 19615) were obtained from the American Type Culture Collection (Manassas, VA, USA). These were used to create confluent plates of colonies on Mueller-Hinton agar for *B. stearothermophilus* and *E. coli* and Mueller-Hinton agar containing 5% sheep blood for *S. pyogenes*. Then 8 mm filter paper disks were dipped in the admission urine samples and pressed onto the agar surface. Duplicate tests for each sample were performed and the plates were incubated overnight at 56 °C for *B. stearothermophilus* and 35 °C for *E. coli* and *S. pyogenes*. The diameters of zones of inhibition were measured and expressed as the mean of the duplicate tests. Zones ≥10 mm with any of the 3 assay strains were considered positive, but results were subject to the following interpretation.

The sensitivities and specificities for the detection of antimicrobial activity from individual plates have been determined as 100.0% and 85.9% respectively for *B. stearothermophilus*, 94.9% and 94.9% respectively for *S. pyogenes* and 71.8% and 98.7% respectively for *E. coli* (Liu *et al.*, 1999). However, the combination of *S. pyogenes* and *E. coli* exhibited a sensitivity of 97.4% and specificity of 94.9% [Liu *et al.*, 1999] and so this was used as the criteria for a positive result indicative of a patient having taken antimicrobial drugs within the previous 48 hours. Samples that produced zones on *B. stearothermophilus* plates only were considered equivocal.

2.2.15 Other Laboratory Investigations

C-reactive protein (CRP), creatine phosphokinase (CPK) and urinary total protein were measured on admission as part of the study. CRP was measured in order to see if it could help distinguish between viral and bacterial infections. This test was done using a simple semi-quantitative latex agglutination assay (Clinotech Diagnostics, Richmond, BC, Canada) that uses serial dilutions to generate results of either <6, 6, 12, 24, 48, 96 or >96 mg/L. CPK and urinary total protein were measured to see if they could help distinguish leptospirosis from other infections. These tests were done using automated analysers at the Nawaloka Hospital, Colombo, Sri Lanka, which is one of the leading private hospitals in the country and runs its own ISO-certified laboratory in conjunction with the Metropolis laboratory chain based in India.

Other haematology and biochemistry parameters were measured as part of the patients' routine management by clinicians on the Professorial Medical Unit and not in a systematic way relating to the study. These investigations were performed either at the Department of Pathology of the Colombo North Teaching Hospital (CNTH) or by a variety of private laboratories adjacent to the hospital. Most of these private laboratories are run by the leading private hospitals in the country and their results are generally considered to be accurate by clinicians at CNTH.

2.3 Formulation of Clinical Prediction Rules for Use in Sri Lanka

Attempts were made to formulate clinical prediction rules for all confirmed infections that had >50 cases diagnosed using gold standard investigations.

2.3.1 Statistical Methods for the Formulation of Clinical Prediction Rules

Data was exported from FileMaker Pro into SPSS 19 (SPSS Inc., Chicago, IL, USA) for statistical analysis. Univariate analysis of binary variables was performed using either Pearson's chi-square test or Fisher's exact test to obtain unadjusted odds ratios (UORs) with 95% confidence intervals (CIs). Univariate analysis of continuous variables was performed using Student's independent samples t-test, which was

interpreted in conjunction with Levene's test for equality of variances and results to obtain UORs with 95% CIs for appropriate unit increases in each variable.

Multivariate analysis using logistic regression was then performed to calculate adjusted odds ratios (AORs) for the variables associated with each infectious disease. However, if the use of complete case methodology for the logistic regression led to >10% of cases being excluded due to missing values, then multiple imputation methodology was used with 5 iterations to produce 5 (complete) imputed data sets for further analysis [Roth & Switzer, 1999]. Logistic regression was then performed by selecting candidate variables with P values > 0.1, which were then eliminated one by one using a backwards stepwise approach. The odds ratios for new model were now AORs, which had been adjusted for the effects of each other.

In order to develop clinical prediction rules, scoring systems using point values were developed from the regression coefficient (labelled "B" in SPSS) in the logistic regression model and receiver operating characteristic (ROC) curves were generated to assess the discriminant ability of the model [McNeil & Hanley, 1984]. Sensitivity and specificity (with 95% CI) were determined using the prediction rule at different cut-off values. Positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard methods.

2.4 Evaluation of Commercial Diagnostic Tests for Use in Sri Lanka

Attempts were made to evaluate commercial diagnostic tests for confirmed infections that had >50 cases diagnosed using gold standard investigations. This was done prospectively for dengue fever because an appropriate test was already available (Section 2.4.1) and the number of confirmed cases was expected to be significantly >50. The evaluation of other commercial diagnostic tests was expected to take place immediately after the study period, but no other suitable tests were available at this time. In due course other rapid diagnostic tests for dengue (Section 2.4.2) and chikungunya (Section 2.4.3) were evaluated retrospectively using the data and samples obtained in this study.

2.4.1 Prospective Use of a Commercial Diagnostic Test for Dengue

Several commercial dengue diagnostic tests were available, which at the time of the study were all serology tests. Those made by Panbio (Panbio Pty Ltd, Brisbane, Australia) seemed to be the most widely used and highly regarded [Vaughn *et al.*, 1999; Groen *et al.*, 2000; Blacksell *et al.*, 2006a; Blacksell *et al.*, 2006b; Blacksell *et al.*, 2007; Blacksell *et al.*, 2008]. The Panbio Dengue Duo Cassette (Catalogue no. R-DEN03D) was a new serology test based on materials and methods evaluated previously. It is an immuno-chromatic test (ICT) packaged in a convenient cassette mechanism for point-of-care testing, which can be used with either whole blood or serum and is read with the naked eye.

In this study the Panbio Dengue Duo Cassette was used to test samples obtained on admission, discharge (if the patient was admitted for >5 days) and follow-up (at 2-4 weeks after discharge) as described in Section 2.1.3. All tests were performed according to the manufacturer's instructions by myself using serum samples on the same day that the blood was taken. The results were determined by naked eye as negative, equivocal or positive for each IgM and IgG band. Samples that initially returned an equivocal result were re-tested to confirm the result.

The presence of an IgM positive band was indicative of acute dengue infection, which was classed as a primary infection if there was no IgG positive band or a secondary infection if there was an IgG positive band. The presence of an IgG band alone was indicative of a previous dengue infection only. Diagnostic accuracy was calculated for the dengue assays relative to the final patient diagnosis (dengue positive or dengue negative) based on the results of the reference tests. However, due to the subjective nature of reading ICTs, equivocal results were first assigned as positive and then as negative for separate analyses to determine which grouping had the best diagnostic accuracy. Diagnostic performance was assessed by calculations of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with exact 95% confidence intervals (CI).

2.4.2 Retrospective Use of Commercial Diagnostic Tests for Dengue

After the main study period in 2006-7, several new commercial diagnostic tests for dengue became available including some that combined IgG & IgM serology with NS1 antigen detection. Therefore six rapid diagnostic tests were evaluated at a later date using the samples and reference diagnostic results obtained for 549 patients from this study.

The six assays were (i) the Biosynex Immunoquick Dengue Fever IgG & IgM Assay (Biosynex, France), (ii) the Merlin Dengue Fever IgG & IgM Combo Device (Merlin), (iii) the Panbio Dengue Duo IgG & IgM Cassette (Inverness, Australia), (iv) the Bio-Rad NS1 Antigen Strip (Bio-Rad, France), (v), the Panbio Dengue NS1 Antigen Strip (Inverness, Australia) and (vi) the Standard Diagnostics Dengue Duo NS1 Antigen and IgG/IgM Combo Device (Standard Diagnostics, South Korea). All assays were performed according to the manufacturers' instructions at the Mahidol-Oxford Tropical Medicine Research Unit (MORU), Bangkok, Thailand by an experienced operator.

Diagnostic accuracy was calculated for these dengue rapid diagnostic tests by comparing the interpretation of the operator to the final patient diagnosis (dengue positive or dengue negative) from the reference serology. Equivocal results where the immuno-chromatographic line was faint were considered negative for the purpose of diagnostic accuracy evaluation. Diagnostic performance was assessed from calculations of sensitivity, specificity, NPV and PPV with exact 95% CIs using Stata/SE 10.0 (Stata Corp., College Station, TX).

2.4.3 Retrospective Use of a Commercial Diagnostic Test for Chikungunya

Also following the main study period, a pair of commercial serology tests for chikungunya became available. Therefore these were evaluated at a later date using the samples and reference diagnostic results obtained for 292 patients with paired serum samples from this study.

The assays were the Standard Diagnostics chikungunya IgM antibody rapid ICT and chikungunya IgM antibody ELISA (Standard Diagnostics, South Korea). All assays were performed according to the manufacturers' instructions at MORU, Bangkok, Thailand by an experienced operator. The ICT was tested with acute specimens only (as would be the case in clinical practice) and the ELISA was tested with both acute and convalescent specimens.

Diagnostic accuracy was calculated for these chikungunya diagnostic tests by comparing the interpretation of the operator to the final patient diagnosis (chikungunya positive or chikungunya negative) from the reference serology. Equivocal results where the immuno-chromatographic line was faint were considered negative for the purpose of diagnostic accuracy evaluation. Diagnostic performance was assessed from calculations of sensitivity, specificity, NPV and PPV with exact 95% CIs using Stata/SE 10.0 (Stata Corp., College Station, TX).

Chapter 3
Results

3.1 Patient Recruitment, Data Collection and Sample Collection

3.1.1 Patient Recruitment

As planned, patient recruitment took place over a 1-year period from 06 June 2006 to 05 June 2007 on the Professorial Medical Unit (PMU), which is responsible for a quarter of all adult medical admissions at the Colombo North Teaching Hospital. During this time there were 11 173 patients admitted, of whom 711 (6.4%) had fever ≥38.0 °C and of these 617 (86.8%) were recruited. The reasons for non-recruitment were :

- "already received antibiotics in hospital" in 42 (44.7%)
- "admitted for >24 hours" in 31 (33.0%)
- "refused consent" in 21 (22.3%)

Recruited patients were compared to both non-recruited patients and all patients admitted with respect to sex, age and date of admission to ensure that recruitment was representative. These data were reviewed on a monthly basis throughout the study so that immediate action could be taken to remedy any problems. A summary of these figures per month is shown (Table 3.1.1.) Admission and recruitment figures for April are artificially low because the PMU was closed to admissions for two weeks during the undergraduate clinical exams.

Amongst all patients admitted there were 53.2% males ($p < 0.01$ compared to females admitted). However, amongst patients with fever there were 64.6% males ($p < 0.01$ compared to males admitted) and amongst patients recruited there were 65.6% males ($p < 0.74$ compared to males with fever). Hence with respect to sex ratio, the recruited population was representative of all patients with fever. However, there was a significant difference in the sex ratio of all patients admitted compared to patients with fever.

Amongst patients with fever the mean age (SD) was 38.0 (16.7) years and was similar for both patients recruited (37.9 (14.0) years) and those not recruited (38.4 (17.2) years). However, amongst males with fever the mean age (SD) was 35.5

(15.5) compared to 42.5 (17.7) in females. There were similar differences between males and females recruited (35.5 (15.2) years *vs.* 42.6 (18.1) years) and males and females who were not recruited (35.7 (17.8) years *vs.* 42.2 (15.8) years). Hence with respect to mean age, the recruited population was representative of all patients with fever. However, there was a significant difference in the mean age of males with fever compared to females with fever.

Although the total number of patients admitted each month was remarkably consistent (Table 3.1.1), the number of patients with fever varied significantly and seemed to be related to monthly rainfall (Figures 3.1.1.1 & 3.1.1.2). The greatest number of patients with fever was seen one month after the rainy seasons in October and April, whereas the least number was seen at the peak of the dry seasons in July and February. The diseases that made the greatest contribution to this seasonal variation were dengue and chikungunya and the monthly frequencies of confirmed cases are shown (Figures 3.1.1.3 & 3.1.1.4). The pattern of rainfall seen during the study period was comparable with that seen in the previous five years, except for an unexpected increase in August and a greater volume of rainfall during the October-November rainy season (Figures 3.1.1.5 & 3.1.1.6).

Table 3.1.1. Patients admitted, patients with fever and patients recruited each month

Month	Jun 2006	Jul 2006	Aug 2006	Sep 2006	Oct 2006	Nov 2006	Dec 2006	Jan 2007	Feb 2007	Mar 2007	Apr 2007	May 2007	Totals
All admitted	952 (100%)	911 (100%)	946 (100%)	947 (100%)	938 (100%)	1008 (100%)	1030 (100%)	938 (100%)	797 (100%)	959 (100%)	783 (100%)	964 (100%)	11,173 (100%)
Males admitted	493 (52%)	435 (48%)	503 (53%)	491 (52%)	522 (56%)	533 (53%)	564 (55%)	485 (52%)	401 (50%)	507 (53%)	424 (54%)	591 (61%)	5,949 (53%) [1]
Females admitted	459 (48%)	476 (52%)	443 (47%)	456 (48%)	416 (44%)	475 (47%)	466 (45%)	453 (48%)	396 (50%)	452 (47%)	359 (46%)	373 (39%)	5,224 (47%)
No. with fever (% of all admitted)	38 (4%)	31 (3%)	56 (6%)	55 (6%)	64 (7%)	117 (12%)	97 (9%)	53 (6%)	42 (5%)	50 (5%)	33 (4%)	75 (8%)	711 (6%)
All with fever	38 (100%)	31 (100%)	56 (100%)	55 (100%)	64 (100%)	117 (100%)	97 (100%)	53 (100%)	42 (100%)	50 (100%)	33 (100%)	75 (100%)	711 (100%)
Males with fever	24 (63%)	26 (84%)	31 (55%)	38 (69%)	47 (73%)	77 (66%)	61 (63%)	33 (62%)	25 (60%)	32 (64%)	19 (58%)	46 (61%)	459 (65%) [2]
Females with fever	14 (37%)	5 (16%)	25 (45%)	17 (31%)	17 (27%)	40 (34%)	36 (37%)	20 (38%)	17 (40%)	18 (36%)	14 (42%)	29 (39%)	252 (35%)
No. recruited (% of all with fever)	22 (58%)	23 (74%)	51 (91%)	42 (76%)	54 (84%)	110 (94%)	83 (86%)	45 (85%)	38 (90%)	47 (94%)	31 (94%)	71 (95%)	617 (87%)
All recruited	22 (100%)	23 (100%)	51 (100%)	42 (100%)	54 (100%)	110 (100%)	83 (100%)	45 (100%)	38 (100%)	47 (100%)	31 (100%)	71 (100%)	617 (100%)
Males recruited	17 (77%)	20 (87%)	29 (57%)	28 (67%)	44 (81%)	74 (67%)	51 (61%)	28 (62%)	24 (63%)	29 (62%)	19 (61%)	42 (59%)	405 (66%) [3]
Females recruited	5 (23%)	3 (13%)	22 (43%)	14 (33%)	10 (19%)	36 (33%)	32 (39%)	17 (38%)	14 (37%)	18 (38%)	12 (39%)	29 (41%)	212 (34%)

[1] $P < 0.01$ compared to females admitted; [2] $P < 0.01$ compared to males admitted; [3] $P = 0.74$ compared to males with fever

Figure 3.1.1.1. Rainfall in Gampaha District, patients admitted and patients with fever during this study in 2006-7

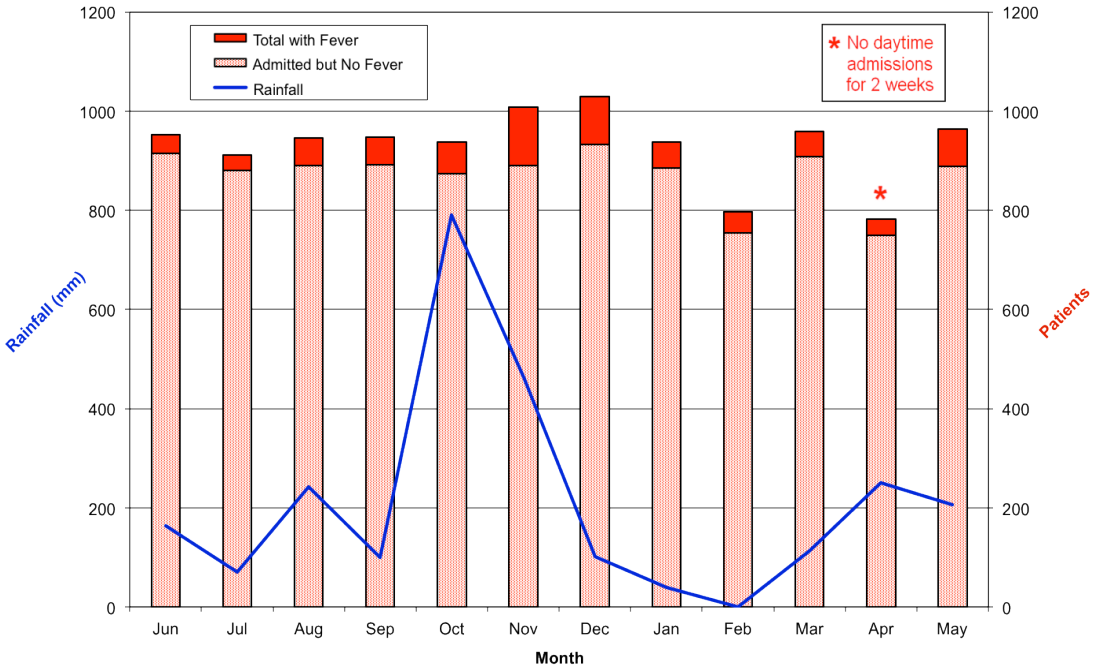


Figure 3.1.1.2. Rainfall in Gampaha District, patients recruited and patients with fever but not recruited during this study in 2006-7

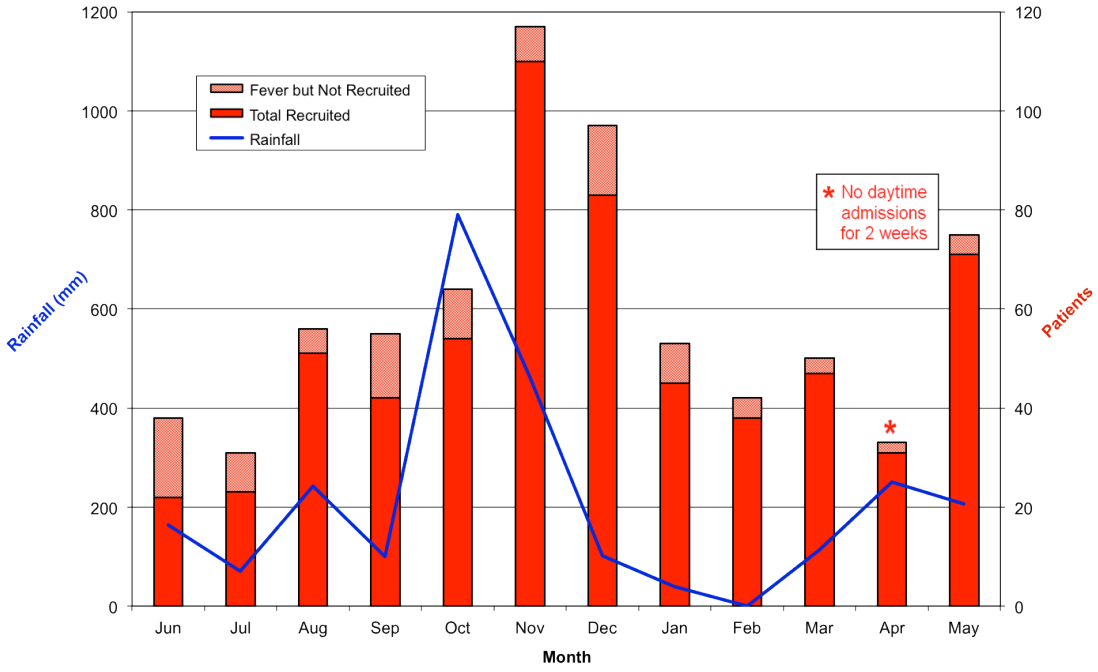


Figure 3.1.1.3. Rainfall in Gampaha District and confirmed dengue cases during this study in 2006-7

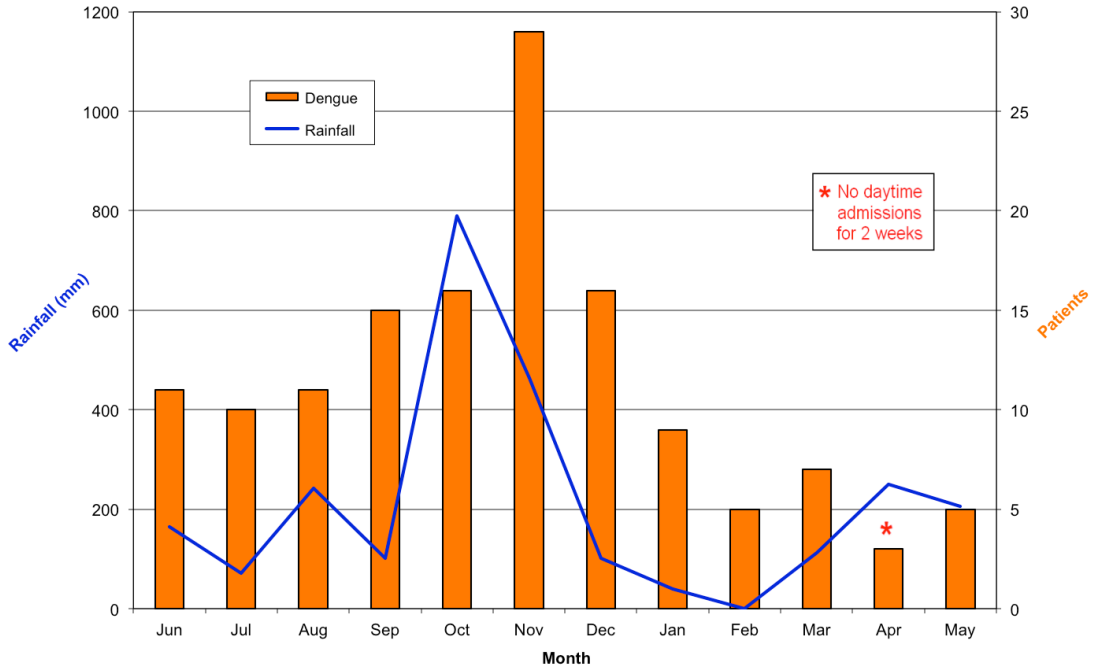


Figure 3.1.1.4. Rainfall in Gampaha District and confirmed chikungunya cases during this study in 2006-7

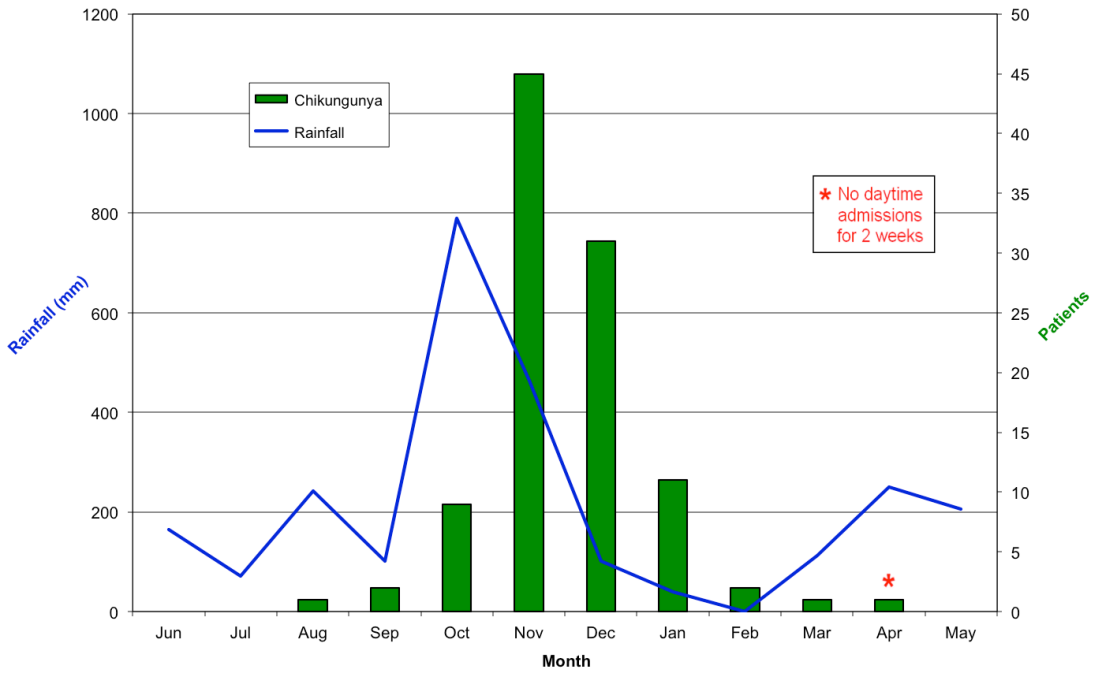


Figure 3.1.1.5. Rainfall in Gampaha District from 2001-7 and mean from 2001-7

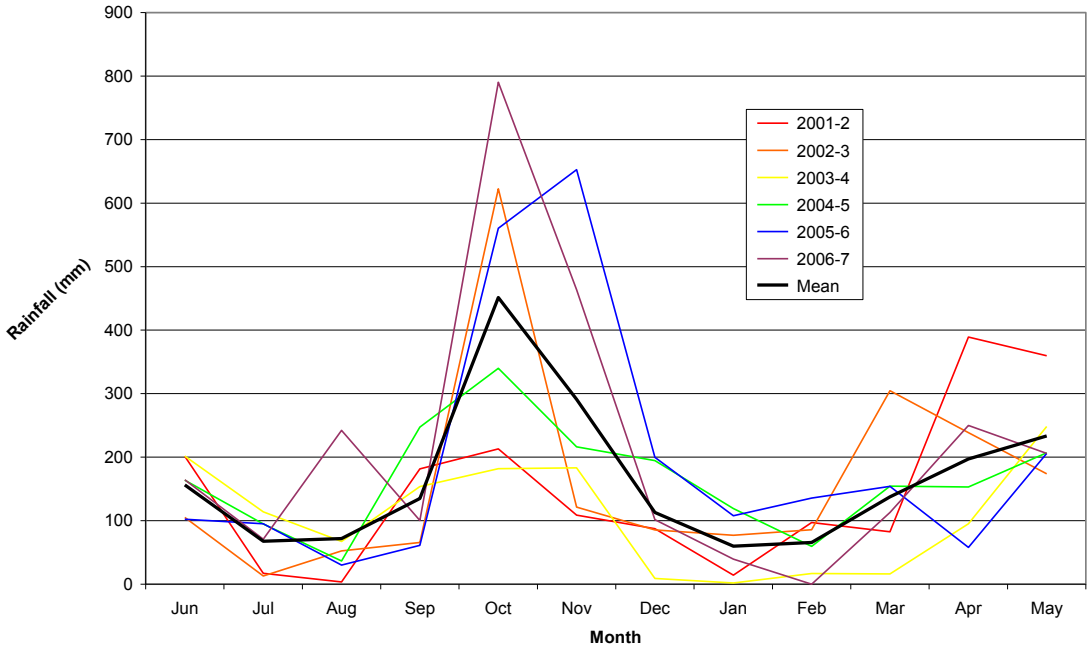
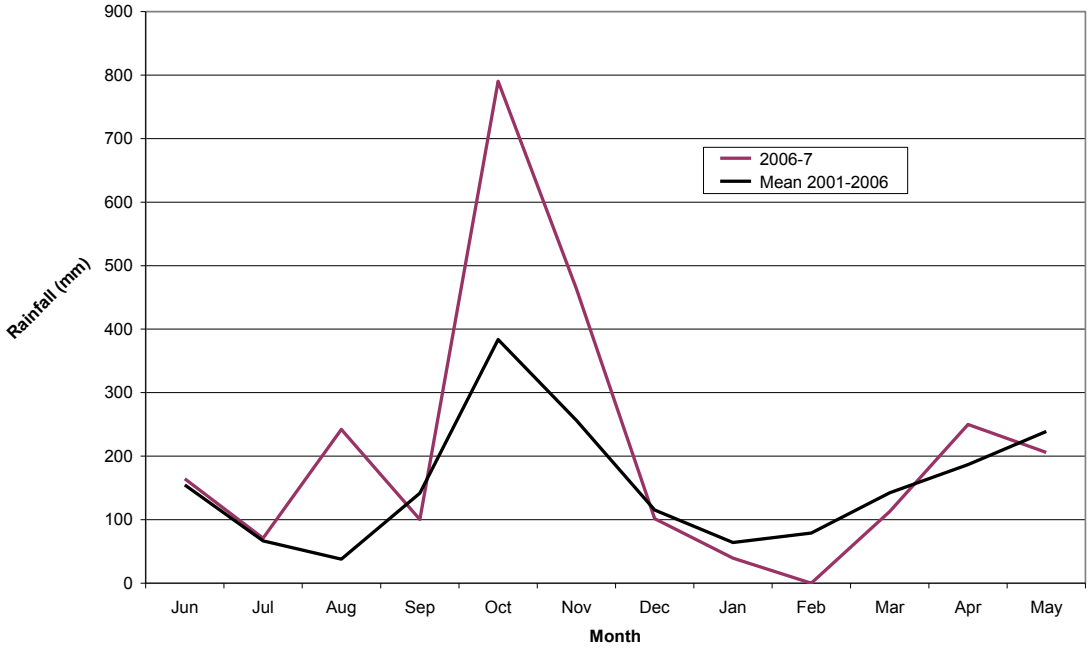


Figure 3.1.1.6. Rainfall in Gampaha District from 2006-7 and mean from 2001-6



3.1.2 Data Collection

All 617 patients recruited had data collection forms completed and the quality of this work seemed to be very good due to the specific training and daily supervision of the research assistants. Data entry and storage in a FileMaker Pro database proved extremely convenient and the electronic back-up strategy avoided any loss of data occurring.

The large amount of epidemiological, clinical and laboratory data collected will be analysed in relation to each disease identified. However, a few epidemiological details for the whole group of recruited patients are worthy of description here.

As reported earlier, 65.6% of patients recruited were male and on average these were significantly younger than the female patients recruited (Section 3.1.1). However, these findings were representative of all patients admitted with fever and hence seem to be genuine. The increased number of fever cases presenting towards the end of the rainy seasons and decreased number presenting at the peak of the dry seasons have also been described earlier (Section 3.1.1) and seem to be a genuine seasonal effect.

Amongst recruited patients the average time spent in education showed a mean (SD) of 10 (3) years or a median (range) of 10 (0-22) years. The average income showed a mean (SD) of LKR 16 000 (13 000) or a median of LKR 12 000 (0 – 100 000) and LKR 100 ≈ US$ 1. The occupations reported were as shown in Table 3.1.2.1.

The districts where people lived are shown in Table 3.1.2.2 and Figure 3.1.2.1. From 548 patients living in the Gampaha District, there were 541 for whom an exact Grama Niladari (GN) division could be identified and these are shown in Figure 3.1.2.2 along with a map of the overall population distribution within Gampaha District in Figure 3.1.2.3. These maps suggest that recruitment was fairly representative of the whole Gampaha District if the background population distribution is taken into account.

Amongst 541 patients with known GN divisions from Gampaha District, there were 221 (41.4%) from urban areas (population density >400 people / km^2) and 320 (59.1%) from rural areas (population density <400 people / km^2).

The range of water sources used for drinking, washing and bathing are shown in Table 3.1.2.3. These findings are consistent with the mixture of urban and rural areas covered in the study and for patients from Gampaha District there was a strongly significant association between the type of area where a patient lived and their source of drinking water as shown in Table 3.1.2.4. Hence the source of a person's drinking water could be used as a practical indicator of whether they lived in an urban or rural area without the need to identify their GN division and calculate its population density.

Notifiable disease reports for 2006, the study period and 2007 are summarised in Table 3.1.2.5. Gampaha District was consistently in the top three districts for cases of dengue, encephalitis and leptospirosis during these periods.

Table 3.1.2.1. Occupations of 617 patients admitted with fever

Occupation	**Number (%)**
House work	81 (13%)
Education	77 (12%)
Construction	70 (11%)
Office work	64 (10%)
Factory work	57 (9%)
Shop work	56 (9%)
Retired	45 (7%)
Transport/Postal work	38 (6%)
Unemployed	37 (6%)
Stone mason	18 (3%)
Hotel work	14 (2%)
Farming	13 (2%)
Military work	12 (2%)
Police/Security work	12 (2%)
Healthcare work	7 (1%)
Religious work	7 (1%)
Fishing	5 (1%)
Unknown	4 (1%)

Table 3.1.2.2. District of origin for 617 patients with fever

District	Number (%)
Gampaha	548 (89%)
Puttalam	24 (4%)
Kurunegala	14 (2%)
Colombo	10 (2%)
Kegalle	7 (1%)
Other	4 (1%)
Unknown	10 (2%)

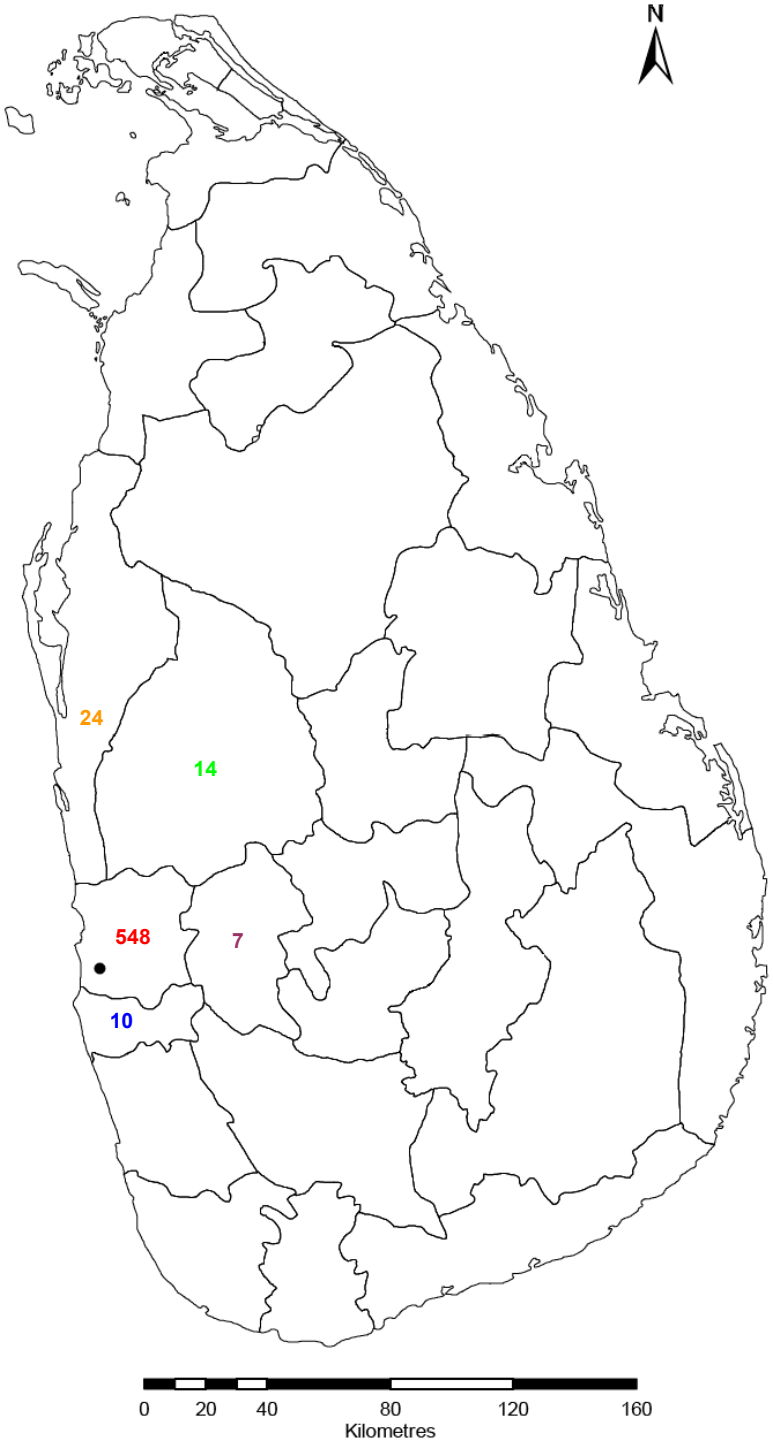
Table 3.1.2.3. Sources of water for 612 patients admitted with fever

	Boiled / Bottled	Tap	Well	"Tank" / Stream
Drinking	63 (10%)	232 (38%)	316 (52%)	1 (0%)
Washing	0 (0%)	255 (42%)	352 (57%)	5 (1%)
Bathing	0 (0%)	250 (41%)	356 (58%)	6 (1%)

Table 3.1.2.4. Association between area of residence and source of drinking water

	Tap (%)	Well (%)	Total (%)
Urban area (≥400 people / km^2)	118 (24%)	80 (17%)	198 (41%)
Rural area (<400 people / km^2)	92 (19%)	195 (40%)	287 (59%)
Total	210 (43%)	275 (57%)	485 (100%)
Chi-squared = 35.084		**P <0.0001**	

Figure 3.1.2.1. District of origin for 617 patients with fever



●	Colombo North Teaching Hospital
548	548 Cases from Gampaha District
24	24 Cases from Puttalam District
14	14 Cases from Kurunegala District
10	10 Cases from Colombo District
7	7 Cases from Kegalle District

Figure 3.1.2.2. Hospital location and patients recruited from each GN division in Gampaha District

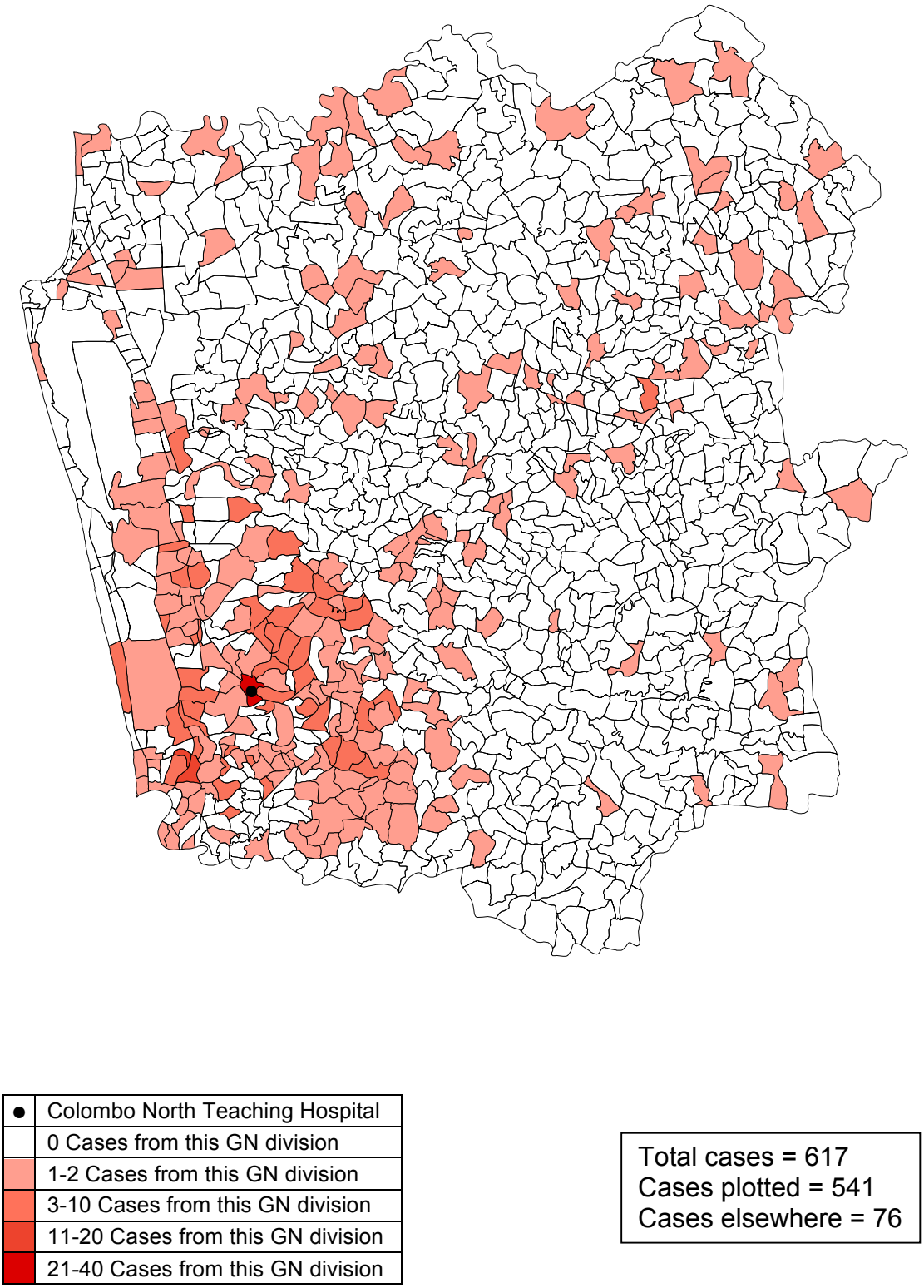
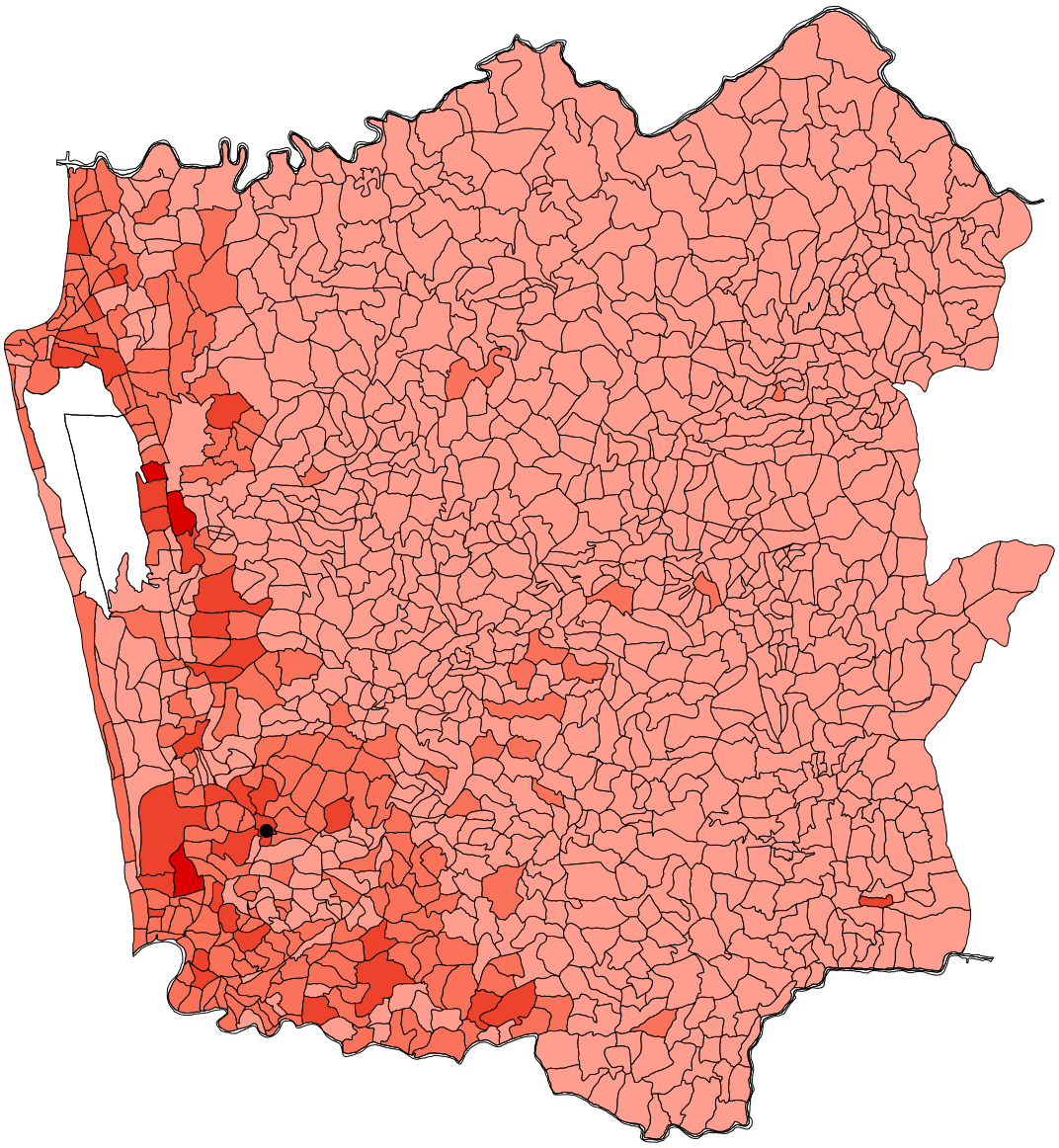


Figure 3.1.2.3. Hospital location and population of each GN division in Gampaha District from the (National) Census in 2001



●	Colombo North Teaching Hospital
	0 People in this GN division
	1-2500 People in this GN division
	2501-5000 People in this GN division
	5001-7500 People in this GN division
	7501-10000 People in this GN division

Total population = 2 060 579

Table 3.1.2.5. *Notifiable diseases reported in Sri Lanka during 2006, this study period and 2007

District	Dengue			Dysentery			Encephalitis			Enteric Fever			Food Poisoning			Leptospirosis			Typhus Fever			Viral hepatitis		
	2006	Study	2007	2006	Study	2007	2006	Study	2007	2006	Study	2007	2006	Study	2007	2006	Study	2007	2006	Study	2007	2006	Study	2007
Colombo	3419	2800	1865	356	371	361	7	6	11	82	83	124	45	78	96	143	140	163	4	2	5	69	65	155
Gampaha	1774	1247	980	331	343	324	10	17	30	59	64	88	147	112	64	211	230	311	12	15	19	153	145	212
Kalutara	977	741	425	506	539	488	8	6	6	84	76	65	72	56	43	136	154	221	6	1	3	47	66	64
Kandy	1509	1414	415	452	361	316	9	10	6	115	85	66	35	22	16	101	94	150	107	98	87	139	978	1975
Matale	389	353	121	344	271	252	7	7	6	19	22	40	21	15	13	31	36	172	2	3	5	30	99	141
Nuwara Eliya	42	60	41	353	304	237	0	0	2	193	153	122	19	377	369	12	15	14	45	50	38	289	418	561
Galle	260	180	100	196	204	173	3	7	13	13	14	26	12	7	44	72	83	169	21	36	27	4	14	24
Hambantota	217	149	102	100	96	200	9	11	6	35	30	24	32	31	20	51	48	57	92	76	74	59	50	29
Matara	537	387	236	199	273	298	11	12	10	81	71	54	36	23	25	176	209	289	253	271	220	15	24	35
Jaffna	53	30	239	141	154	175	4	3	2	176	325	439	27	17	15	3	0	0	141	94	134	78	36	30
Kilinochchi	2	2	1	23	3	1	0	0	0	6	4	7	24	24	0	0	0	0	0	2	2	9	6	4
Mannar	3	9	7	57	55	32	0	0	0	139	63	116	3	3	0	1	0	2	0	0	0	10	8	26
Vavuniya	20	28	41	153	126	86	5	5	4	96	69	21	84	62	65	2	2	3	0	0	0	8	8	14
Mullaitivu	2	3	0	24	19	40	1	5	8	36	18	21	9	1	2	0	0	0	0	0	0	10	2	17
Batticaloa	63	58	79	242	509	477	3	8	12	40	25	24	16	23	10	6	3	0	0	0	22	215	325	1176
Ampara	33	20	5	263	267	204	0	0	0	12	5	6	4	4	2	15	7	8	4	1	3	19	20	37
Trincomalee	42	61	63	218	267	335	1	1	4	45	30	30	42	58	25	3	6	12	4	3	23	175	128	121
Kurunegala	681	637	791	553	658	532	5	3	9	87	87	73	44	43	37	74	64	87	34	44	46	64	51	109
Puttalam	398	397	372	329	295	217	3	11	17	115	75	106	7	5	9	21	31	31	2	0	9	154	90	84
Anuradhapura	131	94	276	446	440	203	8	15	10	44	44	22	28	14	17	47	26	41	27	24	20	64	46	48
Polonnaruwa	84	80	70	155	175	170	1	2	3	10	11	14	124	124	64	22	22	22	5	4	0	23	34	52
Badulla	154	124	83	786	734	651	4	3	7	119	89	99	45	23	13	39	48	49	168	165	169	184	138	400
Monaragala	43	34	57	367	379	363	7	8	2	76	62	60	23	24	40	31	38	56	98	76	93	72	39	47
Ratnapura	542	323	449	758	693	602	19	19	20	110	79	79	39	24	24	79	65	82	38	20	32	189	141	105
Kegalle	481	404	444	279	294	312	3	7	11	59	60	69	16	16	10	289	220	246	62	42	46	396	148	270
Kalmunai	46	9	9	252	280	243	1	2	4	75	31	10	17	15	14	3	2	2	1	2	2	264	144	133
TOTAL	11902	9644	7271	7883	8110	7292	129	168	203	1926	1675	1805	971	1201	1037	1568	1543	2187	1126	1029	1079	2739	3223	5869

*Most diagnoses are based on clinical criteria only.
Red font indicates the three highest incidences per district for each disease during the period indicated.

3.1.3 Sample Collection

From 617 patients recruited, the numbers of diagnostic samples collected and tested were as shown in Table 3.1.3. Serum samples and bacterial isolates were successfully transported to the research and reference laboratories described previously (Section 2.1.3. and 2.2).

Table 3.1.3. Samples collected from 617 patients admitted with fever

Sample	Number	Percentage
In-house blood culture for bacteria	595	96%
BacT/ALERT blood culture for bacteria	604	98%
Any blood culture for bacteria	606	98%
Blood culture for leptospires	604	98%
Blood sample for CRP tests	559	91%
Blood sample for CPK tests	542	88%
Urine sample on admission	472	76%
Serum sample on admission	550	89%
Serum sample on discharge	73	12%
Serum sample on follow-up	300	49%
Any serum sample	573	93%
Single serum sample only	278	45%
Paired serum samples	295	48%

3.2 Laboratory Results

A summary of the diagnoses made from the 617 patients in this study is shown in Table 3.2. These are arranged in groups as follows:

Group 1 diagnoses were confirmed infections in which the exact organism was identified using gold standard laboratory tests.

Group 2 diagnoses were confirmed infections in which the exact organism could not be identified using a gold standard test. Cellulitis and orchitis were diagnosed if clinically obvious. LRTI, sinusitis and PID were diagnosed if there were supportive imaging results. Pulmonary TB was diagnosed according to the "clinically-typical" criteria defined previously (Section 2.2.5). CNS infections were diagnosed if there were raised leukocyte and protein counts in cerebrospinal fluid. Abscesses and septic arthritis were diagnosed by imaging and aspiration.

Group 3 diagnoses were confirmed non-infectious diseases in which the cause was confirmed by gold standard laboratory tests.

Group 4 diagnoses were unconfirmed by any of the above methods and represent the clinical diagnosis made by a senior clinician at the time the patient was discharged from hospital.

There were 230 cases (37.2%) with unconfirmed diagnoses, which were evenly distributed throughout the 12-month study period with the exception of a month following the Sinhalese / Tamil New Year on 14 April 2007. During this period 44 (75.9%) of 58 cases had unconfirmed diagnoses and 23 (52.3%) of these were acute respiratory infections. There were 14 cases (2.3%) who died, of whom five (35.7%) had confirmed non-infectious diseases (two myocardial infarctions, one cerebrovascular accident, one liver failure & one leukaemia), five (35.7%) had leptospirosis (three confirmed & two unconfirmed), three (21.4%) had LRTIs (all unconfirmed) and one (7.1%) had cellulitis (with liver failure).

Table 3.2. Final diagnoses in 617 patients admitted with fever

Group	Definition	Disease	Cases*	%	Total
1	Confirmed infections + organism identified	Dengue	137	22.2%	348 (56.4%)
		Chikungunya	103	16.7%	
		Leptospirosis	32	5.2%	
		Bacteraemia (see Section 3.2.2)	26	4.2%	
		Q fever	18	2.9%	
		Rickettsial infections	14	2.3%	
		Tuberculosis	7	1.1%	
		UTI (no bacteraemia)	5	0.8%	
		Mumps	2	0.3%	
		Chickenpox	1	0.2%	
		Hantavirus	1	0.2%	
		Hepatitis A	1	0.2%	
		Malaria	1	0.2%	
2	Confirmed infections + no organism identified	Cellulitis (clinically obvious)	15	2.4%	47 (7.6%)
		LRTI (with CXR changes)	13	2.1%	
		Pulmonary tuberculosis (see Section 2.2.4)	10	1.6%	
		CNS infection (with CSF changes)	2	0.3%	
		Liver abscess (on imaging & aspiration)	2	0.3%	
		Lung abscess (on imaging & aspiration)	1	0.2%	
		Septic arthritis (on imaging & aspiration)	1	0.2%	
		Sinusitis (with SXR changes)	1	0.2%	
		Orchitis (clinically obvious)	1	0.2%	
		PID (with USS changes)	1	0.2%	
3	Confirmed non-infectious diseases	Malignancy	8	1.3%	22 (3.6%)
		CVA	4	0.6%	
		IBD	2	0.3%	
		MI	2	0.3%	
		Polyarthropathy	2	0.3%	
		SLE	2	0.3%	
		Appendicitis	1	0.2%	
		Pancreatitis	1	0.2%	
4	Unconfirmed diseases	Viral fever	82	13.3%	230 (37.2%)
		Undifferentiated fever	48	7.8%	
		LRTI	33	5.3%	
		UTI	21	3.4%	
		Leptospirosis	17	2.8%	
		URTI	9	1.5%	
		Gastroenteritis	6	1.0%	
		Liver disease	6	1.0%	
		Enteric fever	4	0.6%	
		Acute abdomen	1	0.2%	
		Connective tissue disease	1	0.2%	
		Endocarditis	1	0.2%	
		Malaria	1	0.2%	

*The total number of diagnoses exceeds 617 due to the presence of co-infections.

3.2.1 Malaria Results

From 419 patients who had at least one thick blood film inspected, there was one (0.2%) positive for malaria and also one in whom the diagnosis was made clinically despite negative blood films. The single confirmed case of malaria was diagnosed as having a mixed *Plasmodium falciparum* and *P. vivax* infection and had recently returned from travelling in India, which was thought to be the origin of his infection. Neither of these cases had any other co-infections diagnosed.

3.2.2 Bacteraemia Results

From 606 patients who had at least one blood culture for bacteria done, there were 26 (4.3%) with bacteraemia (Table 3.2.2), of whom one had co-infection with dengue diagnosed.

Bacteraemia was detected in 18/595 (3.0%) in-house blood culture bottles compared to 21/604 (3.5%) in the BacT/ALERT bottles (P = 0.63). This difference was not significant and also did not suggest that the BacT/ALERT bottles were better at identifying bacteria in patients that had antimicrobials in their urine from pre-admission use.

Contamination with non-pathogenic bacteria was found in 63/595 (10.6%) in-house bottles compared to 19/604 (3.1%) in the BacT/ALERT bottles (P < 0.01). This is probably because the in-house bottles had to be opened for sub-culturing as their rubber caps became permanently damaged if a sub-venting needle was left in place and this would prevent them being reused after sterilisation. In comparison the BacT/ALERT bottles remained sealed throughout with a sub/venting needle inserted through their rubber caps and were disposed of after use.

Bacterial identification and antibiotic profile results from the University of Kelaniya, Sri Lanka correlated well with those from the University of Liverpool, UK. Bacterial identifications were the same for 24 (92.3%) of 26 isolates and both exceptions differed at the species level only. Isolate R051 was initially identified as *E. aerogenes* and later confirmed as *E. cloacae*, but these are known to be closely-

related species with only minor differences in their biochemical profiles [Farmer *et al.*, 1985]. Also isolate R222 was initially identified as *B. cepacia* and later confirmed as *B. pseudomallei*, but it is well-known that biochemical profiles can be misleading when trying to distinguish these species [Inglis *et al.*, 2005]. Antibiotic profiles also correlated well between the different laboratories and were identical in 24 (92.3%) of 26 cases.

Acquired antibiotic resistance was identified in six (23.1%) of the 26 isolates obtained from blood cultures. One *S. typhi* isolate was resistant to ampicillin, chloramphenicol, ciprofloxacin and co-trimoxazole. From the *E. coli* isolates, four were resistant to ampicillin, of which two were also resistant to co-amoxiclav and one of these was also resistant to cephalosporins, ciprofloxacin and gentamicin. The *K. pneumoniae* isolate showed extended spectrum beta-lactamase (ESBL) resistance and was confirmed to have the CTX-M-15 enzyme on genetic testing.

Two cases of bacteraemia shown in Table 3.2.2 were of particular interest and could merit publication as case reports. Patient R130 was a 25-year-old woman with Haemoglobin E (HbE) β-thalassemia and a splenectomy, who presented with a severe gastroenteritis and had *Campylobacter jejuni* isolated from her blood cultures. Patient R222 was a 35-year-old paddy farmer with type 1 diabetes mellitus, who presented with a severe LRTI followed by septic arthritis and death, who had *Burkholderia pseudomallei* isolated from his blood cultures. The isolate was initially identified as *B. cepacia* using microbial identification panels in Sri Lanka, but was later identified as *B. pseudomallei* using 16S rRNA gene sequence analysis in the UK. Soil samples from the patient's paddy fields were sent to the Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, Thailand for culture and these grew *B. thailandensis* only. This is usually thought to be an environmental species with minimal pathogenicity, but human infections have been reported previously [Glass *et al.*, 2006].

Table 3.2.2. Cases of bacteraemia diagnosed by blood cultures

Infection	Cases	UI	Organism	IHB	BAB	AMs
UTI	9	R059	*Escherichia coli*	–	+	ND
		R061	*Escherichia coli*	+	+	–
		R249	*Escherichia coli*	–	+	–
		R350	*Escherichia coli*	–	+	+/–
		R390	*Escherichia coli*	+	–	ND
		R459	*Escherichia coli*	–	+	+/–
		R507	*Escherichia coli*	–	+	–
		R608	*Citrobacter kosseri*	–	+	+
		R051	*Enterobacter cloacae*	+	–	–
LRTI	6	R021	*Streptococcus pneumoniae*	+	+	–
		R481	*Streptococcus pneumoniae*	+	–	+
		R526	*Streptococcus pneumoniae*	+	+	ND
		R222	*Burkholderia pseudomallei*	+	+	+
		R609	*Enterobacter cloacae*	+	+	+
		R419	*Klebsiella pneumoniae*	–	+	–
Enteric Fever	5	R353	*Salmonella typhi*	+	–	+
		R427	*Salmonella typhi*	+	+	+/–
		R431	*Salmonella typhi*	+	+	–
		R518	*Salmonella typhi*	+	+	ND
		R519	*Salmonella typhi*	+	+	+
Abscess	2	R472	*Acinetobacter calcoaceticus*	–	+	+
		R181	*Staphylococcus aureus*	+	+	–
Endocarditis	2	R471	*Streptococcus bovis*	+	+	+
		R479	*Streptococcus viridans*	+	+	+
Gastroenteritis	1	R130	*Campylobacter jejuni*	+	+	ND
URTI	1	R502	*Streptococcus pyogenes*	+	–	ND
Totals	**26**			**18**	**21**	

UTI, urinary tract infection; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection; UI, unique identifier; IHB, in-house bottle; BAB, BacT/ALERT bottle; AMs, antimicrobials detected in urine; ND, not done; +/–, equivocal.

3.2.3 Other Bacterial Results

Other bacterial infections in urine and sputum samples were diagnosed as part of the patients' normal management, but very few samples were sent to the Department of Microbiology, University of Kelaniya. (It seems likely that more were sent to the Department of Pathology of the Colombo North Teaching Hospital instead, but very few of these could be traced.) From 617 patients in the study, there were five (0.8%) who had urinary tract infections (UTIs) diagnosed from urine samples alone and none who had lower respiratory tract infections (LRTIs) diagnosed from sputum samples alone. None of these five UTI cases had any co-infections diagnosed. All other cases of UTI or LRTI were diagnosed by blood cultures due to the associated bacteraemias (Section 3.2.2).

3.2.4 Tuberculosis Results

From 617 patients in the study, there were 17 (2.8%) diagnosed as tuberculosis (TB). There were seven with laboratory-confirmed TB (three on culture and four on histology) of whom five had pulmonary TB and two had extra-pulmonary TB. There were also 10 with "clinically-typical" pulmonary TB as defined previously (Section 2.2.4) of whom five had good responses to anti-tuberculous therapy and five had undocumented responses due to their care being transferred away from the (National) Chest Hospital at Welisara. All patients diagnosed with TB had raised inflammatory markers with ESR ≥65 mm/h or CRP ≥96 mg/L, which exceeded the necessary levels defined in Section 2.2.4. None of these 17 TB cases had any co-infections diagnosed.

3.2.5 Brucellosis Results

From 573 patients who provided serum samples (295 paired samples and 278 single samples), there were five (0.8%) positive at a dilution of 1:160 on the Brucellacapt screening assay. The complete brucellosis serology results for these patients are shown in Table 3.2.5.

Although EIA IgM titres >20 are considered positive, MAG titres of 20 to 160 are of indeterminate clinical significance and none of these five patients had significant EIA IgG or CFT titres. Hence none of them have evidence of acute or chronic brucellosis, nor is it possible to speculate regarding previous exposure to *Brucella* in these patients.

Table 3.2.5. Complete brucellosis serology results for patients with a positive screening result

Sample	Brucellacapt	EIA IgM	EIA IgG	MAG	CFT
R056A	Positive	40	<20	80	<4
R469A	Positive	<20	<20	ND	ND
R476F	Positive	<20	<20	ND	ND
R503F	Positive	<20	<20	ND	ND
R529F	Positive	20	<20	40	<4

3.2.6 Leptospirosis Results

From 604 patients who had a blood culture for leptospires done, there were 19 (3.1%) that had *Leptospira* isolated. From 573 patients who provided serum samples (295 paired samples and 278 single samples), there were 35 (6.1%) positive on the IgM ELISA, but only 23 (4.0%) positive on the MAT. Combining the culture and ELISA results would identify 41 cases of leptospirosis, whereas combining the culture and MAT results would identify only 32 cases. These results are summarised in Table 3.2.6.1. Of the nine cases not proven on gold standard investigations (culture or MAT), seven had equivocal MAT results and seven had either a 4-fold rise in their IgM ELISA titre or else the maximum possible titre (1:2,560) on admission and only one (R590) had neither of these results. So the number of leptospirosis cases confirmed by gold standard tests remains 32, but there is evidence to suggest that the true number of confirmed cases should be 40 or 41. In addition there were 8 unconfirmed cases, who were clinically diagnosed with leptospirosis but not fully investigated with cultures and paired serology tests and in three this was due to early death or urgent transfer for haemodialysis. Of the 32 confirmed leptospirosis cases, three had co-infections diagnosed of whom one had dengue, one had Q fever and one had scrub typhus.

CAAT and MLST analysis was performed on 14 (73.7%) and 17 (89.5%) of the 19 isolates because the remainder could not be recovered from frozen storage. These results have been entered in the *Leptospira* MLST website database (http://leptospira.mlst.net) as shown in Table 3.2.6.2 and have also been submitted to GenBank [Boonsilp *et al.*, 2013]. The CAAT analysis identified nine of 14 isolates as Pyrogenes at serogroup and serovar level and the remaining five isolates had uncertain identities. The MLST analysis was more specific and identified sequence types (STs) for all 17 isolates and revealed eight different sequence types (STs). However, four of these (STs 49, 74, 75 and 76) were closely related within a single clonal complex (CC 12 in this case) and these accounted for 13 (76.5%) of the 17 isolates identified. Overall, these results are suggestive of endemic disease and not an outbreak situation in which a dominant clone predominates, as was reported in Thailand [Thaipadungpanit *et al.*, 2007].

Some of the clinical and laboratory features on admission for confirmed leptospirosis cases from this study are shown in Table 3.2.6.3. The characteristic appearance of conjunctival suffusion with icterus in a leptospirosis patient (R561) is shown in Figure 3.2.6.1, but this is a very rare occurrence in Sri Lanka since most cases an anicteric. With only 32 confirmed cases it was thought that performing multiple logistic regression and developing a clinical prediction rule would not be feasible. However, samples to evaluate leptospirosis diagnostic tests are available from 32 confirmed cases, of whom 23 have paired serum samples (Table 3.2.6.1) and 17 have had isolates identified by CAAT or MLST (Table 3.2.6.2).

Of all the infections tested for, leptospirosis was the most likely to have a focal distribution due to its mode of transmission and so the distribution of the 41 cases identified by culture or ELISA were plotted as shown in Figure 3.2.6.2. One GN division (Ihala Karagahamuna North with a population of 3634 on the Census in 2001) had three cases (R025, R028 & R503), but cultures were negative for all of these and so a comparison of isolates was not possible. The remaining cases had a similar distribution to the other patients recruited (Figure 3.1.2.2) and the overall population of Gampaha District (Figure 3.1.2.3).

Table 3.2.6.1. Cases of leptospirosis diagnosed by blood culture and serology tests

UI	Culture	Serum	ELISA	MAT	AMs
R010	+	Paired	+	+	–
R025	–	Paired	+	+/–	+
R026	–	Paired	+	+/–	+
R028	–	Single	+	+/–	+
R065	–	Paired	+	+	+
R116	+	Paired	+	+	–
R122	+	Single	+/–	+	–
R136	–	Paired	+	+	+
R144	–	Paired	+	+/–	+
R150	+	Paired	+	+/–	+
R160	ND	Paired	+	+	ND
R163	+	Paired	+	+	–
R166	+	Single	–	–	ND
R168	+	Single	–	–	ND
R186	+	Paired	+	–	–
R194	–	Paired	+	+	+
R205	+	Paired	+	+	–
R235	+	Paired	+	+	–
R238	+	Paired	+	+	ND
R248	–	Paired	+	–	–
R312	–	Single	+	+/–	ND
R358	+	Single	–	–	+/–
R407	–	Single	+	+	+
R437	+	Paired	+	+	+
R444	+	Single	–	–	+/–
R450	–	Paired	+	+	ND
R457	+	Paired	+	+	+/–
R461	–	Single	+	+/–	+
R474	–	Paired	+	+	+
R480	+	Paired	+	–	ND
R489	–	Paired	+	+	+
R493	+	Single	+	+	ND
R499	+	Paired	+	+	+
R503	–	Paired	+	+	+
R554	–	Paired	+	+	+
R561	–	Paired	+	+/–	+/–
R567	–	Paired	+	+	+
R590	–	Paired	+	–	+/–
R597	–	Single	+	+	+
R601	+	Single	–	–	–
R612	–	Paired	+	+	–

UI, unique identifier; ELISA, enzyme-linked immuno-sorbent assay; MAT, micro-agglutination test; AMs, antimicrobials detected in urine; ND, not done; +/–, equivocal. Shading indicates cases that were not proven by gold standard investigations (culture or MAT).

Table 3.2.6.2. CAAT and MLST analysis of isolates from the Ragama Fever Study in the *Leptospira* MLST Database (http://leptospira.mlst.net)

Case	Host	Gender	Age	Country	Region	Latitude	Longitude	Year	Species	Serogroup	Serovar	ST	glmU	pntA	sucA	tpiA	pfkB	mreA	caiB	Source
R010	Human	Male	49	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. borgpetersenii*	Possibly Celledoni	Undesignated	144	24	27	30	34	67	27	28	Mark Bailey
R116	Human	Female	45	Sri Lanka	Kurunegala	7.4833333	80.3666667	2006	*L. borgpetersenii*	Undesignated	Undesignated	157	28	27	29	28	40	26	14	Mark Bailey
R122	Human	Male	17	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. interrogans*	Pyrogenes	Pyrogenes	49	5	1	1	1	3	2	7	Mark Bailey
R150	Human	Male	20	Sri Lanka	Kegalle	7.1204053	80.3213106	2006	*L. interrogans*	Pyrogenes	Pyrogenes	49	5	1	1	1	3	2	7	Mark Bailey
R163	Human	Male	44	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. interrogans*	Pyrogenes	Pyrogenes	74	1	1	1	2	3	2	7	Mark Bailey
R166	Human	Male	30	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. interrogans*	Possibly Canicola	Undesignated*	49	5	1	1	1	3	2	7	Mark Bailey
R168	Human	Male	38	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. interrogans*	Pyrogenes	Pyrogenes	49	5	1	1	1	3	2	7	Mark Bailey
R205	Human	Male	29	Sri Lanka	Puttalam	8.0341667	79.8352778	2006	*L. interrogans*	Pyrogenes	Pyrogenes	75	5	1	1	5	3	2	7	Mark Bailey
R235	Human	Male	24	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. interrogans*	Possibly Grippotyphosa	Undesignated	140	3	3	3	3	4	5	16	Mark Bailey
R358	Human	Male	53	Sri Lanka	Gampaha	7.089895	79.999413	2006	*L. interrogans*	Pyrogenes	Pyrogenes	49	5	1	1	1	3	2	7	Mark Bailey
R437	Human	Male	24	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Died	Died*	76	5	1	1	2	3	2	7	Mark Bailey
R444	Human	Male	24	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Died	Died*	75	5	1	1	5	3	2	7	Mark Bailey
R457	Human	Male	51	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Died	Died*	76	5	1	1	2	3	2	7	Mark Bailey
R480	Human	Male	24	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Pyrogenes	Pyrogenes	49	5	1	1	1	3	2	7	Mark Bailey
R493	Human	Male	42	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Pyrogenes	Pyrogenes	75	5	1	1	5	3	2	7	Mark Bailey
R499	Human	Male	60	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Possibly Hebdomadis	Undesignated	80	3	1	17	3	4	5	16	Mark Bailey
R601	Human	Male	54	Sri Lanka	Gampaha	7.089895	79.999413	2007	*L. interrogans*	Pyrogenes	Pyrogenes	75	5	1	1	5	3	2	7	Mark Bailey

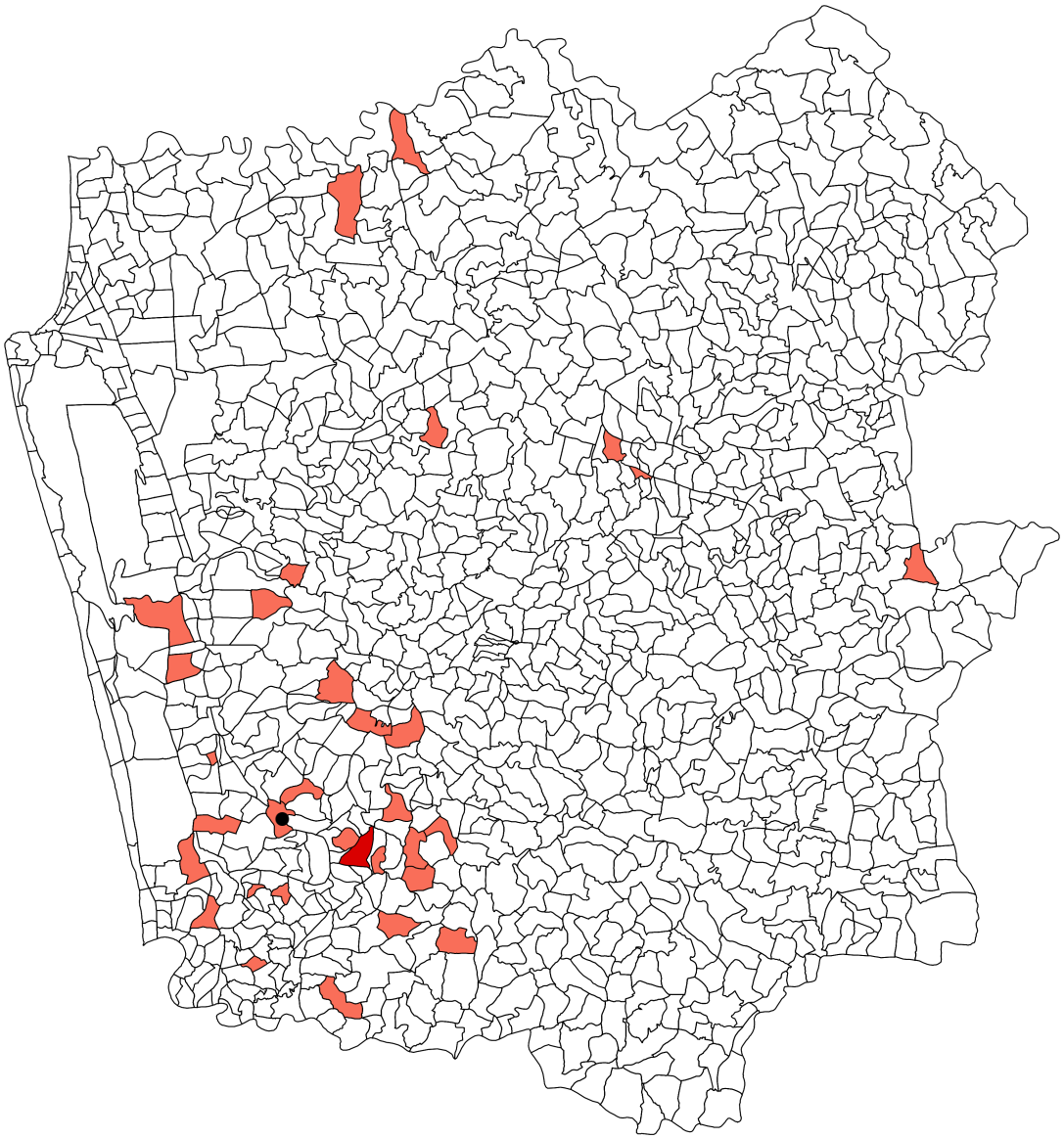
* Probably Pyrogenes based on ST; Isolates shown in shaded boxes are all from CC 12; Isolates shown in colour are unique to Sri Lanka

Table 3.2.6.3. Clinical and laboratory features on admission for confirmed leptospirosis cases from this study

History	%	Examination	%	Investigations	%
Male sex	95	Conjunctival suffusion	41	Creatinine > 1.50 mg/dL	85
Chills	93	Hepatomegaly	22	Urea > 25 mg/dL	83
Myalgia	88	Photophobia	20	Platelets < 150 x 10^9/L	62
Headache	85	Hypotension	17	SGOT (AST) > 40 IU/L	46
Arthralgia	83	Oliguria	15	Platelets < 100 x 10^9/L	41
Rigors	66	Flushing	12	Proteinuria > 100 mg/dL	39
Vomiting	49	ITU admission	12	Neutrophils > 8.0 x 10^9/L	35
Anorexia	39	Icterus/jaundice	10	SGPT (ALT) > 60 IU/L	29
Dysgeusia	34	Bleeding	7	Lymphocytes < 1.0 x 10^9/L	27
Sore throat	32	Lymphadenopathy	7	WCC > 4.0 x 10^9/L	22
Cough	29	Splenomegaly	2	Bilirubin > 20 md/dL	6
Abdominal pain	27	Conjunctival haemorrhage	0	Haematocrit > 0.45	6
Nausea	22	Rash	0		
Diarrhoea	20	Tourniquet test positive	0		
Death	12				

Figure 3.2.6.1. Characteristic (but rare) appearance of conjunctival suffusion with icterus in a leptospirosis patient (R561)

Figure 3.2.6.2. Distribution of leptospirosis cases in Gampaha District



●	Colombo North Teaching Hospital
	0 Cases from this GN division
	1 Case from this GN division
	2 Cases from this GN division
	3 Cases from this GN division

Total cases = 41
Cases plotted = 34
Cases elsewhere = 7

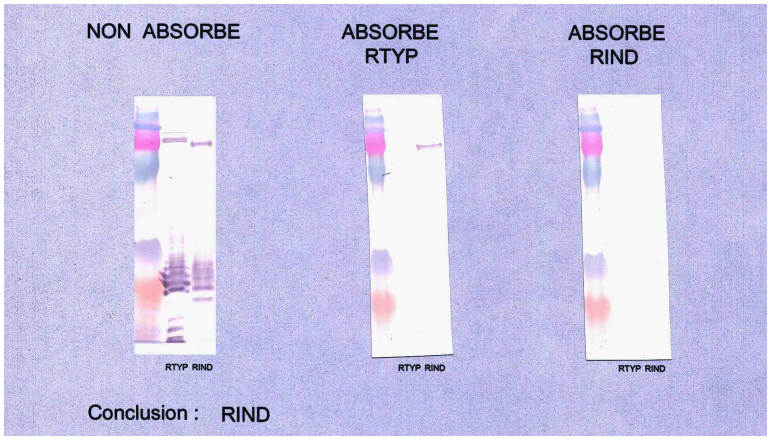
3.2.7 Q fever Results

From 573 patients who provided serum samples (295 paired samples and 278 single samples), there were 18 (3.1%) with Q fever, of whom 13 had acute Q fever, four had probable Q fever and one had chronic Q fever. There were also 23 patients with evidence of past Q fever infection. Of the 18 Q fever cases, nine had co-infections diagnosed of whom five had dengue, one had chikungunya, one had leptospirosis, one had spotted fever and one had probable rickettsial infection.

3.2.8 Rickettsia Results

From 573 patients who provided serum samples (295 paired samples and 278 single samples), there were 14 (2.4%) with rickettsial infections, of whom six had scrub typhus, six had probable rickettsial infection and two had spotted fever, of which one (R527) was identified as *Rickettsia conorii* subspecies *indica* infection by western blotting with cross-adsorption (Figure 3.2.8.1). Of the 14 rickettsial infection cases, five had co-infections diagnosed of whom two had Q fever, two had chikungunya and one had leptospirosis. Amongst the six scrub typhus cases identified in Marseilles, only one had an eschar, only one had a rash and only two were also positive on scrub typhus serology in Bangkok. A total of six patients had eschars, of whom only one was diagnosed as scrub typhus in Marseilles compared to five in Bangkok, but combining these positive results would have diagnosed all six.

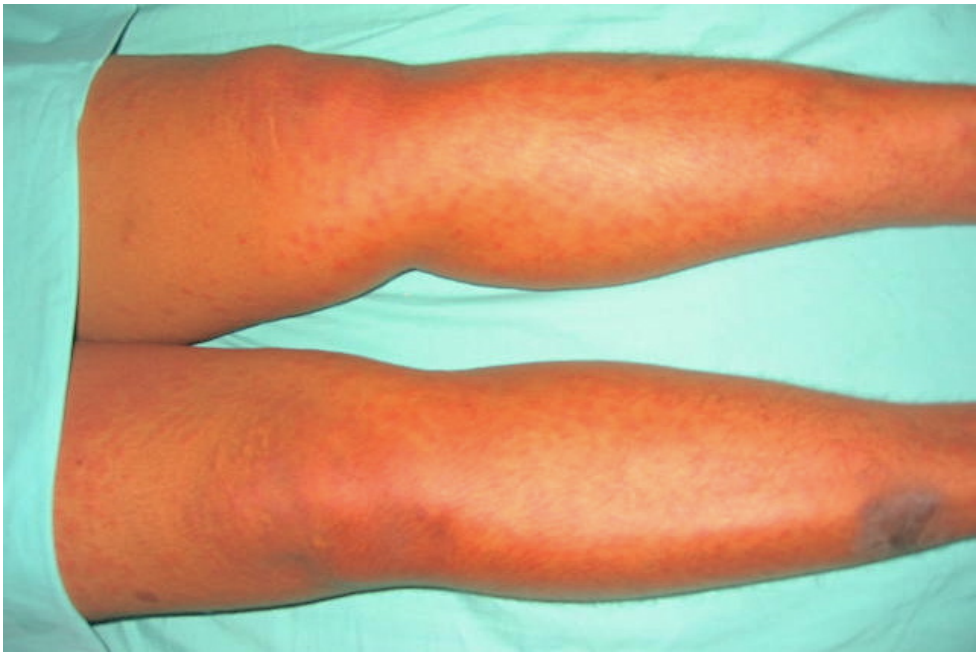
Figure 3.2.8.1. Western blotting with cross-adsorption to distinguish *R. conorii* subspecies *indica* from *R. typhi* in a patient (R527)



The characteristic eschar of scrub typhus (case R349) and a typical rash of rickettsial spotted fever (case R527) are shown below in Figures 3.2.8.2 & 3.2.8.3.

Figure 3.2.8.2. Characteristic appearance of an eschar on the left inner thigh of a scrub typhus patient (R349)

Figure 3.2.8.3. Typical appearance of a rash on the lower limbs of a rickettsial spotted fever patient (R527)



3.2.9 Dengue Results

From 573 patients who provided serum samples (295 paired samples and 278 single samples), there were 109 positive on dengue serology, of whom 20 (18.3%) were acute primary infections, 81 (74.3%) were acute secondary infections, five (4.6%) were recent infections and three (2.8%) were of indeterminate duration. Dengue RT-PCR was performed on admission samples only for dengue seropositive cases and dengue seronegative cases that provided admission serum only. From these samples there were 93 positive on dengue RT-PCR, of whom two (2.2%) were DENV-1, 26 (28.0%) were DENV-2, 62 (66.7%) were DENV-3 and three (3.2%) were DENV-4 on genotyping. The combination of 101 acute dengue serology cases and 93 dengue RT-PCR positive cases resulted in 137 cases of acute dengue infection being identified (Table 3.2.9.1). Therefore from 573 patients tested, there were 137 (23.9%) with acute dengue infection. Of these cases, 18 (13.1%) had co-infections diagnosed of whom 10 had chikungunya infection, three had Q fever, two had probable Q fever, two had leptospirosis and one had bacteraemia due to a urinary tract infection with *E. coli*.

The monthly frequency of dengue cases was shown previously (Figure 3.1.1.3). Some of the clinical and laboratory features on admission for confirmed cases from this study are shown in Table 3.2.9.3. The characteristic (but rare) appearance of a confluent rash with “islands of sparing” in a dengue patient who had DHF (R006) is shown in Figure 3.2.9.1. Data recorded on admission was later used to develop clinical prediction rules for the diagnosis of dengue (Section 3.3.1). From these 137 cases of acute dengue infection, there were 75 with paired serum samples and 49 of these had the serotype identified on RT-PCR testing (Table 3.2.9.2). These samples will be the most useful for evaluating dengue diagnostic tests in the future (Section 3.4).

Table 3.2.9.1. Dengue cases from serology and RT-PCR results

Dengue Serology Tests	Positive	109 (19%)	60/109 = RT-PCR +ve on admission
	Negative	464 (81%)	33/464 = RT-PCR +ve on admission [1]
	Not Done	44	no serum provided
	Total	617	
Dengue Serology	Acute Primary	20 (18%)	15/20 = RT-PCR +ve on admission
	Acute	81 (74%)	42/81 = RT-PCR +ve on admission
	Recent	5 (5%)	1/5 = RT-PCR +ve on admission
	Indeterminate	3 (3%)	2/3 = RT-PCR +ve on admission
	Total	109	
Dengue RT-PCR Tests [2]	Positive	93 (28%)	60/93 = serology +ve
	Negative	242 (72%)	47/242 = serology +ve
	Not Done	282	2/282 = serology +ve [3]
	Total	617	
Dengue RT-PCR	DENV-1	2 (2%)	
	DENV-2	26 (28%)	
	DENV-3	62 (67%)	
	DENV-4	3 (3%)	
	Total	93	
Serology Positive and/or RT-PCR Positive		142	
		-4	Serology = "Recent" and RT-PCR –ve
		-1	Serology = "Indeterminate" and RT-PCR
		137	
		137/573 = 24% of patients tested had acute dengue	

1. Serology negative due to lack of follow-up serum samples.

2. Dengue RT-PCR tests at AFRIMS were performed for dengue seropositive cases and dengue seronegative cases that provided admission serum only. Therefore dengue seronegative cases with paired serum samples (and cases with no serum provided) did not have dengue RT-PCR tests.

3. RT-PCR not done due to lack of admission serum samples.

Table 3.2.9.2. Paired samples available for evaluating dengue diagnostic tests

Serotype	Acute primary	Acute Secondary	Totals
DENV-1	0	1	1
DENV-2	1	11	12
DENV-3	10	26	36
DENV-4	0	0	0
Sub-Totals	**11**	**38**	**49**
Unknown	3	23	26
Totals	**14**	**61**	**75**

Table 3.2.9.3. Clinical and laboratory features on admission for confirmed dengue cases from this study

History	%	Examination	%	Investigations	%
Chills	94	Flushing	41	SGOT (AST) > 40 IU/L	58
Headache	81	Hypotension	17	Platelets < 150 x 10^9/L	51
Myalgia	70	Bleeding	15	SGPT (ALT) > 60 IU/L	47
Male sex	69	Hepatomegaly	15	WCC < 4.0 x 10^9/L	46
Arthralgia	68	Lymphadenopathy	15	Neutrophils < 2.0 x 10^9/L	36
Rigors	64	Conjunctival suffusion	8	Urea > 25 mg/dL	31
Vomiting	62	Photophobia	7	Lymphocytes < 1.0 x 10^9/L	26
Dysgeusia	55	Rash	5	Platelets < 100 x 10^9/L	26
Nausea	34	Splenomegaly	4	Bilirubin > 20 mg/dL	21
Cough	31	Tourniquet test positive	3	Haematocrit > 0.45	19
Diarrhoea	30	Conjunctival haemorrhage	1	Proteinuria > 100 mg/dL	13
Anorexia	28	Icterus/jaundice	1	Creatinine > 1.50 mg/dL	0
Abdominal pain	16	Oliguria	1		
Sore throat	14	ITU admission	1		
Death	0				

Figure 3.2.9. Characteristic (but rare) appearance of a confluent rash with “islands of sparing” in a dengue patient with DHF (R006)

3.2.10 Japanese Encephalitis Results

From 573 patients who provided serum samples (295 paired samples and 278 single samples), for serological testing at AFRIMS, there were no cases of Japanese encephalitis (JE) diagnosed. This is an important result for the Gampaha District of Sri Lanka because it has the highest recorded annual incidence of "encephalitis" (Table 3.1.2.5), high rates of JE exposure have previously been described in humans and animals [Peiris *et al.*, 1993] and the area is well-known for its marshes where mosquito vectors breed and its pig-farming that provides reservoir / amplifying hosts for Japanese encephalitis virus (JEV). For these reasons Gampaha District has had a childhood JE vaccination programme in place since 1988 and although the patients in this study were nearly all too old to have been vaccinated, there may well have been some "herd immunity" conferred to reduce the incidence of JE in the adult population.

3.2.11 Chikungunya Results

From 573 patients who provided serum samples (295 paired samples and 278 single samples), there were 91 positive on chikungunya serology, of whom 52 (57.1%) were acute infections, 12 (13.2%) were recent infections and 27 (29.7%) were of indeterminate duration. Chikungunya RT-PCR was performed on all samples and from the admission samples tested there were 97 positive on chikungunya RT-PCR. The combination of 52 acute chikungunya serology cases and 97 chikungunya RT-PCR positive cases resulted in 103 cases of acute chikungunya infection being identified (Table 3.2.11). Therefore from 573 patients tested, there were 103 (18.0%) with acute chikungunya infection. Of these cases, 13 (12.6%) had co-infections diagnosed, of whom 10 had dengue, two had scrub typhus and one had Q fever.

The testing of all serum samples by chikungunya RT-PCR produced some interesting results. From 73 discharge samples, eight (11.0%) were positive and from 299 follow-up samples, 24 (8.0%) were positive. Of the eight positive cases on discharge, all had other confirmed diagnoses (two had dengue, two had leptospirosis, one had scrub typhus, one had tick typhus/spotted fever, one had cellulitis and one had lymphoma) and had been in hospital for ≥1 week. These features suggest that the

chikungunya infections may have occurred during their hospital stay. From the 24 positive cases on follow-up, five were also positive on admission, were diagnosed as acute chikungunya on serology and had no co-infections. Looking at the dates of their samples shows that these five cases had RT-PCR detectable chikungunya viraemia lasting from 13 to 27 days.

Analysis of the RT-PCR sequence for the chikungunya E1 gene of selected cases and reference isolates was used to generate a phylogenetic tree (Figure 3.2.11). This shows that the chikungunya viruses identified in this study were all closely related to each other and to the epidemic strain circulating in the Indian Ocean area from 2006 onwards [Pialoux *et al.*, 2007]. This strain is related to a central/east African strain and is quite distinct from previous Asian strains.

The monthly frequency of chikungunya cases was shown previously (Figure 3.1.1.4). Some of the clinical and laboratory features on admission for confirmed cases from this study are shown in Table 3.2.11.2. The typical appearance of conjunctival suffusion in a chikungunya patient (R483 - who did not have leptospirosis) is shown in Figure 3.2.11.2. Persistent arthralgia for >2 weeks was found in 27 (52.9%) of 51 cases who attended for follow-up and this is known to be a characteristic feature of the disease. Data recorded on admission was later used to develop clinical prediction rules for the diagnosis of chikungunya (Section 3.3.2). From these 103 cases of acute chikungunya infection, there were 52 with paired serum samples, which will be the most useful for evaluating chikungunya diagnostic tests in the future (Section 3.4).

Table 3.2.11.1. Chikungunya results from serology and RT-PCR results

Chikungunya Serology Tests	Positive	91 (16%)	57/91 = RT-PCR +ve on admission
	Negative	483 (84%)	40/483 = RT-PCR +ve on admission [1]
	Not Done	44	no serum provided
	Total	617	
Chikungunya Serology Positives	Acute	52 (57%)	46/50 = RT-PCR +ve on admission
	Recent	12 (13%)	0/12 = RT-PCR +ve on admission
	Indeterminate	27 (30%)	11/27 = RT-PCR +ve on admission ∴considered
	Total	91	
Chikungunya RT-PCR on Admission [2]	Positive	97 (18%)	57/97 = sero +ve
	Negative	453 (82%)	29/453 = sero +ve
	Not Done	67	5/67 = sero +ve [3]
	Total	617	
Chikungunya RT-PCR on Discharge	Positive	8 (11%)	
	Negative	65 (89%)	
	Not Done	544	
	Total	617	
Chikungunya RT-PCR on Follow-Up	Positive	24 (8%)	
	Negative	275 (92%)	
	Not Done	318	
	Total	617	
Serology +ve and/or RT-PCR +ve on Admission		131	
		-12	Serology = "Recent" and RT-PCR –ve
		-16	Serology = "Indeterminate" and RT-PCR –ve [4]
		103	
		103/573 = 18% of patients tested had acute chikungunya fever	

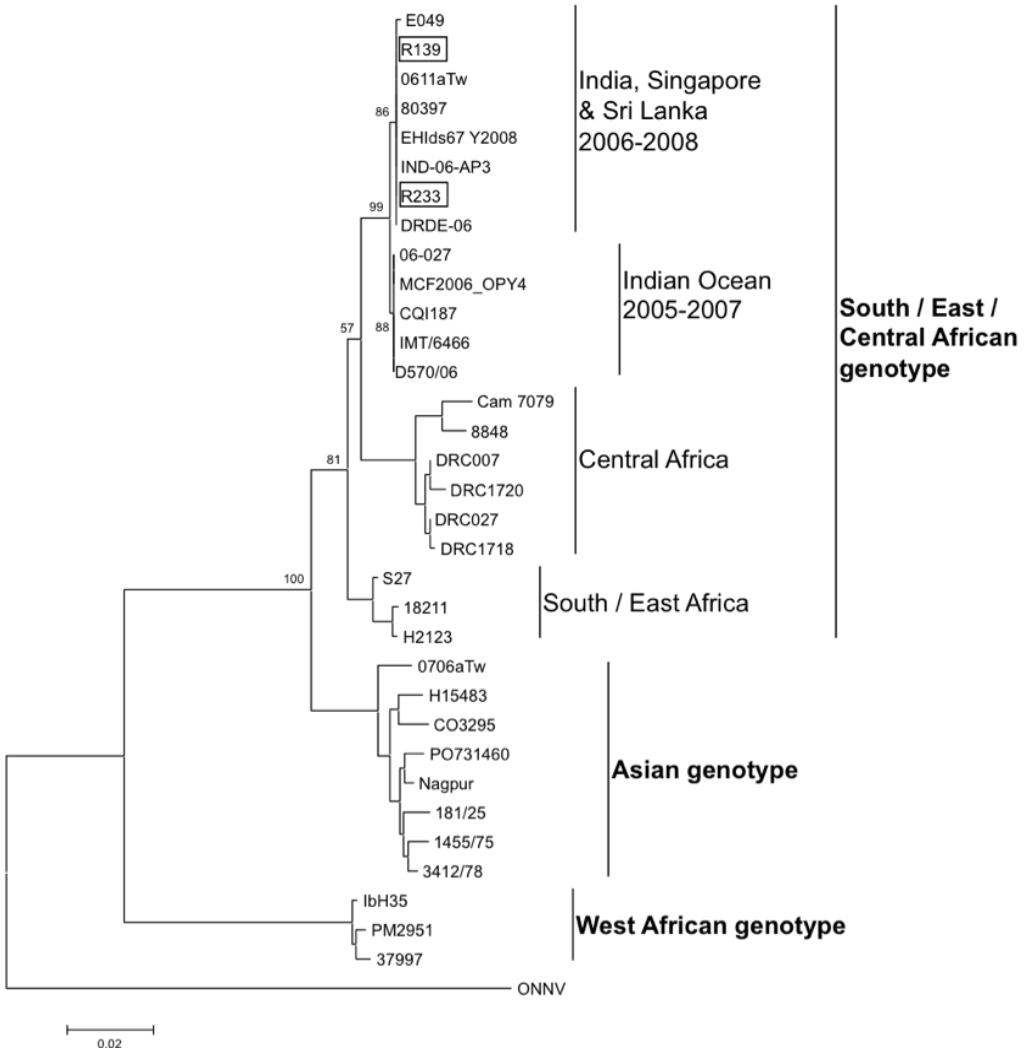
1. Serology negative due to lack of follow-up serum samples.

2. Chikunguya RT-PCR tests were performed for every serum sample and not just for chikungunya seropositive cases and chikungunya seronegative cases that provided admission serum samples only. This strategy raises the possibility that cases with paired serum samples could be seronegative but RT-PCR positive on admission, which would represent conflicting results and be difficult to explain. This did occur in 20 cases despite repeat testing and these were considered to be false positives. Hence the threshold for RT-PCR positivity was adjusted using these cases as false positives and cases with paired serum samples that were seropositive and also RT-PCR positive as true positives. This adjustment classified the conflicting results as RT-PCR negative and also reclassified another 21 positive RT-PCR results as negative.

3. RT-PCR not done due to lack of admission serum samples.

4. There is a high proportion of chikungunya seropositive cases, which can not be classified as "acute" or "recent" because only a single serum sample was provided and chikungunya RT-PCR was negative. From these 16 cases there are 12 that seem to be typical acute chikungunya cases based on their epidemiological, clinical and laboratory data and also very high chikungunya IgM levels.

Figure 3.2.11.1. Phylogenetic tree comparing the partial E1 gene sequences of chikungunya viruses from Sri Lanka (R139 & R233) and reference isolates



Phylogenetic analysis of CHIKV sequences based on partial E1 gene sequences (position 10344 to 10957 of the prototype CHIKV S27 genomic sequence). Sequences obtained in this study are boxed. The analysis was performed using MEGA 4 software [Tamura et al., 2007], using the neighbour-joining (maximum composite likelihood) method. The length of the tree branches indicates the percentage of divergence; the percentage of successful bootstrap replicates is specified at the nodes (1000 replicates). ONNV (O'nyong-nyong virus) prototype sequence was included to root the tree; all other sequences are CHIKV.

Table 3.2.11.2. Clinical and laboratory features on admission for confirmed chikungunya cases from this study

History	%	Examination	%	Investigations	%
Chills	94	Flushing	31	Urea > 25 mg/dL	51
Headache	86	Conjunctival suffusion	20	SGOT (AST) > 40 IU/L	42
Arthralgia	83	Hepatomegaly	14	Platelets < 150 x 10^9/L	33
Myalgia	79	Photophobia	8	Lymphocytes < 1.0 x 10^9/L	29
Male sex	72	Lymphadenopathy	7	SGPT (ALT) > 60 IU/L	23
Rigors	72	Bleeding	5	WCC < 4.0 x 10^9/L	18
Vomiting	47	Hypotension	5	Haematocrit > 0.45	12
Anorexia	37	Rash	4	Neutrophils < 2.0 x 10^9/L	11
Cough	26	Icterus/jaundice	2	Platelets < 100 x 10^9/L	4
Nausea	21	Oliguria	1	Proteinuria > 100 mg/dL	4
Dysgeusia	18	Splenomegaly	1	Bilirubin > 20 mg/dL	0
Diarrhoea	12	Conjunctival haemorrhage	0	Creatinine > 1.50 mg/dL	0
Abdominal pain	7	ITU admission	0		
Sore throat	7	Tourniquet test positive	0		
Death	0				

Figure 3.2.11.2. Typical appearance of conjunctival suffusion in a chikungunya patient (R483 - who did not have leptospirosis)

3.2.12 Hantavirus Results

From 295 patients who provided paired serum samples for serological and RT-PCR tests at NaDP, there were 25 (8.5%) with positive or equivocal results on the Hantavirus IgG DxSelect screening ELISA, of whom most were already positive on admission (Table 3.2.12). From these 25 cases, there were 18 cases positive on the serology gold standard IgM/IgG IFA for SEOV and weaker results (consistent with cross-reactions) were obtained with the equivalent IgM/IgG IFAs for HTNV and PUUV. Six cases (R158, R446, R489, R504, R511 and R590) were also positive on the Hantavirus IgM DxSelect ELISA suggesting acute infection, but only one case (R504) had a greater than four-fold rise in the IFA titre between the admission and follow-up samples to confirm this.

The diagnostic RT-PCR in-house test results were all negative, but this was considered to be unreliable due to a known detection limit of 10,000 viral copies. RT-PCR amplification of the hantavirus S, M and L genome segments with degenerate primers for hantaviruses and specific primers for THAIV hantavirus was performed on the acute serum of case R504. This produced short sequences from the M and L segments only (due to limited amounts of viral RNA being present in the sample).

The M segment sequence was 399 nucleotide bases long and showed 83% similarity to THAIV hantavirus :

TTGTATAATTGGGACTGTATCTAAGTTTATCCAGGGTGATACATTACTTTT
TCTAGGCCCATTAGAAGGTGGTGGTCTAATATTTAAACACTGGTGCACAT
CTACATGTCAGTTTGGTGACCCAGGTGACATCATGAGTCCAAAAAACAA
ACCTTTGCTATGCCCAGATTTCCCGGGTCAGTTTAGGAAAAAATGTAATT
TTGCAACAACCCCTATTTGTGAGTATGATGGTAACAGGATTTCTGGTTAT
AGAAAGATGATGGCTACAATTGATTCTTTCCAATCTTTCAACACAAGTGA
TATTCATTTCACTGATGAACGGATAGAGTGGAAAGACCCTGATGGGATGC
TCCGTGACCATATAAATGTATTGGTTACAAAAGATATTGATTTTGAAAA

The L segment sequence was much shorter at 129 bases :

AAGAATGTTTATAAAACAGACTTTTTCATGTCACGAAAACTGCGTTCCTA
TATTGATTCTATGGAAGGTCTCGAACAGAATGTAAAGGAATTTCTTGAAT
TTTTTCCTGACGGTCATCATGGTGAAGTT

The tree in Figure 3.2.12 shows that the hantavirus from case R504 is most closely related to THAIV, followed by SEOV and then HTNV, but was far-removed from PUUV and TPMV. These M and L sequences were quite distinct from those obtained for the hantavirus previously isolated from rats in Sri Lanka (Sri R 87-1315), which was confirmed to be more closely related to SEOV [JW Song, personal communication].

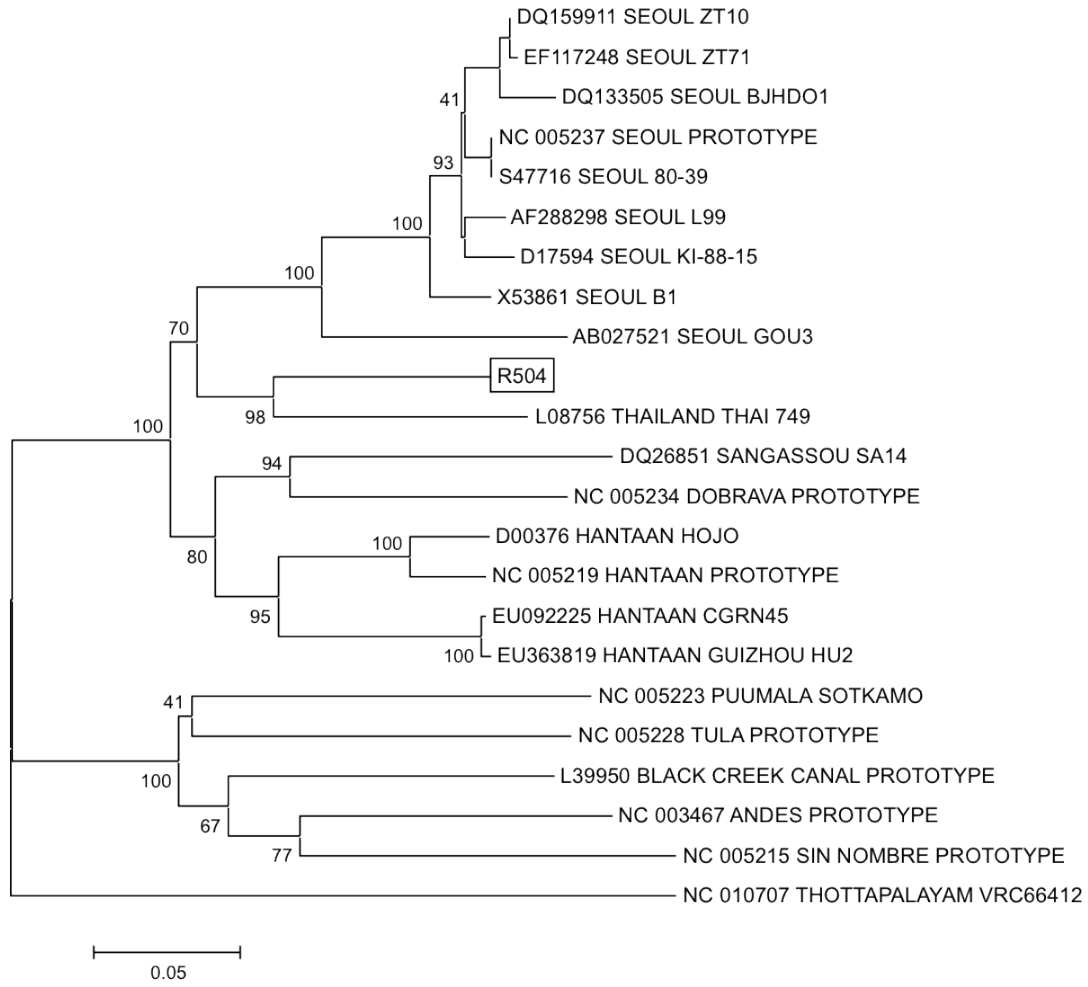
It is interesting to note that case R504 was a 38-year old mechanic with reported close rodent exposure and a previous splenectomy, who presented with a 3-day history of non-productive cough, bilateral lung crepitations, low CRP and had no other confirmed diagnosis made from blood cultures (including those for *Leptospira*) or paired serology tests for all the infections identified in this study. Hence a genuine hantavirus infection seems likely.

Table 3.2.12. Hantavirus results for patients with paired sera that had positive or equivocal results on an IgG screening ELISA

UI	Sample	HANV IgG ELISA 1	HANV IgG ELISA 2	HANV IgM ELISA	SEOV IgM/IgG IFA	HTNV IgM/IgG IFA	PUUV IgM/IgG IFA
R017	A	EQV	POS	POS	NEG	ND	ND
	D	POS	POS	POS	ND	ND	ND
	F	POS	POS	POS	NEG	NEG	NEG
R019	A	POS	POS	NEG	POS	ND	ND
	D	POS	POS	NEG	ND	ND	ND
	F	POS	POS	NEG	POS (+)	NEG	NEG
R094	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (++)	POS (++)	NEG
R111	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (+)	NEG	NEG
R150	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (+)	NEG	NEG
R158	A	NEG	NEG	POS	POS	ND	ND
	D	NEG	NEG	NEG	ND	ND	ND
	F	EQV	EQV	POS	POS (+)	NEG	NEG
R189	A	EQV	EQV	NEG	NEG	ND	ND
	F	POS	POS	NEG	NEG	NEG	NEG
R198	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (++)	POS (++)	POS (+)
R205	A	EQV	EQV	NEG	POS	ND	ND
	F	EQV	EQV	NEG	POS (+)	NEG	NEG
R236	A	EQV	NEG	NEG	POS	ND	ND
	F	POS	EQV	NEG	POS (+)	NEG	NEG
R241	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (++)	POS (++)	NEG
R353	A	EQV	POS	NEG	POS	ND	ND
	F	EQV	POS	NEG	POS (+)	NEG	NEG
R374	A	POS	POS	NEG	NEG	ND	ND
	F	POS	POS	NEG	NEG	NEG	NEG
R390	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	EQV	POS (+)	NEG	NEG
R425	A	NEG	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (+)	NEG	NEG
R446	A	POS	POS	POS	POS	ND	ND
	F	POS	POS	POS	POS (+)	NEG	NEG
R457	A	NEG	NEG	NEG	NEG	ND	ND
	D	EQV	EQV	NEG	ND	ND	ND
	F	POS	EQV	NEG	NEG	NEG	NEG
R458	A	POS	POS	NEG	POS	ND	ND
	F	POS	POS	NEG	POS (+)	NEG	NEG
R479	A	POS	POS	NEG	NEG	ND	ND
	D	POS	POS	EQV	NEG	NEG	NEG
R489	A	POS	POS	POS	POS	ND	ND
	F	POS	POS	POS	POS (++)	POS (+)	NEG
R504*	A	POS	POS	POS	POS	ND	ND
	F	POS	POS	POS	POS (++)	POS (++)	POS (+)
R511	A	POS	EQV	POS	POS	ND	ND
	D	EQV	NEG	POS	ND	ND	ND
	F	POS	POS	POS	POS (+)	NEG	NEG
R530	A	POS	EQV	NEG	NEG	ND	ND
	F	POS	POS	NEG	NEG	NEG	NEG
R588	A	NEG	POS	NEG	NEG	ND	ND
	D	EQV	POS	POS	NEG	NEG	NEG
R590	A	POS	POS	POS	POS	ND	ND
	F	POS	POS	NEG	POS (+)	NEG	NEG

*UI, unique identifier; A, admission; D, discharge; F, follow-up; +, weak positive; ++, strong positive; *, greater than four-fold increase in SEOV IFA titre between admission and follow-up samples.*

Figure 3.2.12 Phylogenetic tree comparing the M segment sequences of a hantavirus from Sri Lanka (R504) and reference isolates



Phylogenetic analysis of hantavirus sequences based on partial M segment sequences. The sequence obtained in this study is boxed. The analysis was performed using MEGA 4 software [Tamura et al., 2007], using the neighbour-joining (p-distance) method. The length of the tree branches indicates the percentage of divergence; the percentage of successful bootstrap replicates is specified at the nodes (1000 replicates). Thottapalayam virus sequence was included to root the tree; all sequences are hantaviruses.

3.2.13 Other Virology Results

For two patients with suspected mumps and one patient with suspected varicella, the diagnosis was confirmed on serology tests at HPA reference labs in the UK.

3.2.14 Urinary Antimicrobial Results

From 472 patients that provided admission urine samples, there were 249 (52.8%) with antimicrobial activity against *B. stearothermophilus*, 159 (33.7%) against *S. pyogenes* and 121 (25.6%) against *E. coli*. Combining the results for *S. pyogenes* and *E. coli* (to maximise the sensitivity and specificity of the test) showed anti-microbial activity in 203 (43.0%) of cases. This left an additional 91 (19.3%) cases with anti-microbial activity against *B. stearothermophilus* only, which were classified as equivocal. However, it indicates that anti-microbial activity may have been present in as many as 294 (62.3%) cases.

3.2.15 Other Laboratory Results

From 559 patients that provided samples for C-reactive protein (CRP) tests, the results were as shown (Table 3.2.15.1).

From 88 patients with confirmed bacterial infections and no co-infections that provided samples for CRP tests (36 had leptospirosis, 22 had bacteraemia, nine had Q fever, nine had rickettsial infections, seven had tuberculosis and five had urinary tract infections), the CRP results were as shown (Table 3.2.15.1).

From 199 patients with confirmed viral infections and no co-infections that provided samples for CRP tests (111 had dengue, 84 had chikungunya, two had mumps, one had chickenpox and one had hepatitis A), the CRP results were as shown (Table 3.2.15.1).

Table 3.2.15.1. CRP results on admission for all cases, confirmed bacterial cases and confirmed viral cases

CRP (mg/L)	All Cases (%)	Bacterial Cases (%)	Viral Cases (%)
<6	140 (25%)	11 (13%)	77 (39%)
6	50 (9%)	1 (1%)	27 (14%)
12	89 (16%)	2 (2%)	50 (25%)
24	90 (16%)	8 (9%)	26 (13%)
48	83 (15%)	15 (17%)	18 (9%)
96	105 (19%)	49 (56%)	1 (1%)
>96	2 (0%)	2 (2%)	0 (0%)
Totals	559 (100%)	88 (100%)	199 (100%)

After excluding co-infections, scatter plots suggested that on admission CRP $\leq$ 12 mg/L was predictive of viral infection and CRP $\geq$ 48 mg/L was predictive of bacterial infection and so these cut-offs were used for further analysis. CRP was $\leq$ 12 mg/L in 154 (77.4%) of 199 viral infections compared to 14 (15.9%) of 88 bacterial infections (OR [95% CI] = 18.089 [8.945 - 37.133], $P < 0.001$) as shown in Table 3.2.15.2. CRP was $\geq$ 48 mg/L in 66 (75.0%) bacterial infections compared to 19 (9.5%) viral infections (OR [95% CI] = 28.421 [13.769 - 59.491], $P < 0.001$) as shown in Table 3.2.15.3.

Table 3.2.15.2. Association between CRP ≤ 12 mg/L on admission and viral or bacterial infections

	Viral Infection	**Bacterial Infection**	**Total**
CRP ≤ 12 mg/L	154	14	168
CRP > 12 mg/L	45	74	119
Total	199	88	287

Diagnostic indices (95% CI) : OR = 18.089 (8.945 - 37.133), P < 0.001; Sensitivity = 0.774 (0.738 - 0.801); Specificity = 0.841 (0.760 - 0.902); PPV = 0.917 (0.874 - 0.949); NPV = 0.622 (0.562 - 0.667); LR+ = 4.864 (3.080 – 8.191); LR- = 0.269 (0.221 - 0.344).

Table 3.2.15.3. Association between CRP ≥ 48 mg/L on admission and bacterial or viral infections

	Bacterial Infection	**Viral Infection**	**Total**
CRP ≥ 48 mg/L	66	19	85
CRP < 48 mg/L	22	180	202
Total	88	199	287

Diagnostic indices (95% CI) : OR = 28.421 (13.769 - 59.491), P < 0.001; Sensitivity = 0.750 (0.672 - 0.812); Specificity = 0.905 (0.870 - 0.932); PPV = 0.776 (0.696 - 0.841); NPV = 0.891 (0.857 - 0.918); LR+ = 7.885 (5.182 - 11.971); LR- = 0.276 (0.201 - 0.376).

There were 377 patients that provided samples on admission for creatinine phosphokinase (CPK) tests and 472 that provided samples on admission for urinary total protein measurements, which were of interest with regard to the diagnosis of leptospirosis. There was no significant association between raised CPK on admission and leptospirosis (Table 3.2.15.4), but there was a marked association between proteinuria (>100 mg/dL) on admission and leptospirosis (Table 3.2.15.5).

Table 3.2.15.4. Association between CPK on admission and leptospirosis

	Leptospirosis	**Not Leptospirosis**	**Total**
CPK >200 U/L	13	102	115
CPK ≤200 U/L	26	236	262
Total	39	338	377
χ^2 = 0.05	**P = 0.83**	**OR (95%CI) = 1.16 (0.54 – 2.46)**	

Table 3.2.15.5. Association between proteinuria on admission and leptospirosis

	Leptospirosis	**Not Leptospirosis**	**Total**
Proteinuria	13	59	72
No Proteinuria	19	381	400
Total	32	440	472
χ^2 = 15.05	**P < 0.01**	**OR (95%CI) = 4.42 (1.94 – 10.00)**	

3.3 Formulation of Clinical Prediction Rules for Use in Sri Lanka

Dengue and chikungunya were each responsible for >50 confirmed cases and so attempts were made to formulate clinical prediction rules for these infections. The number of confirmed leptospirosis cases fell short of this target, which was probably due to diagnostic tests that were impeded by pre-admission antibiotic use or else required convalescent blood samples that were often not available.

3.3.1 Clinical Prediction Rule for the Diagnosis of Dengue

Variables recorded on admission that were potential predictors for a confirmed diagnosis of dengue were first subjected to univariate analysis. This included the use of Pearson's chi square test, Fisher's exact test and odds ratios for categorical variables and Student's independent samples t-test (with 2-tailed significance and Levene's test) for continuous variables. Using these tests the following variables were found to have unadjusted associations ($p < 0.05$) with dengue :

- Decreasing age
- Increasing income
- Flushing
- Dysgeusia
- Hypotension
- Lack of haemoptysis
- Vomiting
- Arthralgia
- Leukopenia
- Thrombocytopenia
- Decreasing CRP
- Increasing SGOT
- Increasing education
- Early presentation to hospital
- Haemorrhage
- Headache
- Lack of cough
- Nausea
- Diarrhoea
- Lack of anaemia
- Neutropenia
- Decreasing ESR
- Increasing SGPT
- Increasing CPK

However, there was apparent overlap between these variables and so multivariate analysis using logistic regression was performed. Unfortunately, the use of complete case methodology led to 48.0% of all cases being excluded due to missing values and hence concerns regarding the representativeness of this analysis. Even when variables with >10% missing values were excluded, there were still >20% of all cases excluded from this type of analysis. Although these analyses of complete cases may have identified adjusted associations that were correct, there are also ethical issues regarding the omission of such a large amount of patient data.

Therefore a multiple imputation method was used with 5 iterations, which allowed the logistic regression to be repeated on the imputed data set (which then included 100% of cases). This identified some variables (occurrence of an eschar,

haemoptysis, or neurological signs) with no occurrences in the dengue cohort and so cases with these features were excluded from the remainder of this analysis (leaving 594 cases to be analysed) and so too were these variables themselves. These clinical features could be used to exclude dengue in this cohort of patients, but only occurred rarely (eschar in six cases, haemoptysis in 16 and neurological signs in one) and both haemoptysis and neurological signs have been described previously in complicated forms of dengue. Some of these features may have occurred with a larger sample size (and more complicated forms of dengue) and would then have been included in the logistic regression.

The results of the final logistic regression on the imputed data sets are shown in Tables 3.3.1.1 and 3.3.1.2. In Table 3.3.1.1 the first row (labelled "Original data") shows that only 310 (52%) of the remaining 594 cases from the original data set would have been included in this analysis, compared to 100% of cases in the imputed datasets. In Table 3.3.1.2 the third column (labelled "S.E") shows that no variables in the pooled imputation analysis had very high standard errors in this analysis, which indicated that the model was stable.

Table 3.3.1.1. Classification table for logistic regression of all dengue predictors

				Predicted		
				Dengue Confirmed		
Imputation Number		Observed		No	Yes	Percentage Correct
Original data	Step 1	Dengue Confirmed	No	220	11	95.2
			Yes	17	62	78.5
		Overall Percentage				91.0
1	Step 1	Dengue Confirmed	No	439	18	96.1
			Yes	50	87	63.5
		Overall Percentage				88.6
2	Step 1	Dengue Confirmed	No	434	22	95.2
			Yes	53	84	61.3
		Overall Percentage				87.4
3	Step 1	Dengue Confirmed	No	434	22	95.2
			Yes	54	83	60.6
		Overall Percentage				87.2
4	Step 1	Dengue Confirmed	No	438	19	95.8
			Yes	53	84	61.3
		Overall Percentage				87.9
5	Step 1	Dengue Confirmed	No	434	22	95.2
			Yes	53	84	61.3
		Overall Percentage				87.4

a. The cut value is .500

Table 3.3.1.2. Logistic regression for full adjusted model for dengue

Imputation Number		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper	Fraction Missing Info.	Relative Increase Variance	Relative Efficiency
Pooled	Step 1[a,b] Age	-.015	.012			.192	.985	.963	1.008	.083	.087	.984
	Male	-.626	.415			.131	.535	.237	1.206	.022	.022	.996
	PopulationDensity	-.001	.000			.282	.999	.999	1.000	.161	.179	.969
	Education	.046	.052			.370	1.047	.947	1.159	.018	.018	.996
	Income	.000	.000			.284	1.000	1.000	1.000	.110	.118	.978
	PipedDrinkingWater	-.443	.318			.164	.642	.344	1.198	.036	.037	.993
	DelayedPresentation	.017	.022			.431	1.017	.975	1.061	.039	.040	.992
	AntibioticsPreAdmission	.663	.355			.063	1.940	.965	3.901	.093	.098	.982
	Smoking	.336	.431			.436	1.399	.601	3.258	.044	.045	.991
	Alcohol	-.216	.361			.550	.806	.397	1.636	.066	.068	.987
	Temperature	.132	.224			.555	1.141	.736	1.769	.041	.042	.992
	FeverIntermittent	-.375	.303			.216	.687	.380	1.245	.054	.055	.989
	Chills	.118	.604			.846	1.125	.344	3.679	.063	.065	.988
	Rigors	.708	.308			.021	2.031	1.111	3.713	.016	.016	.997
	Wound	.107	.654			.870	1.113	.308	4.015	.073	.076	.986
	Flushing	.942	.329			.004	2.566	1.345	4.896	.043	.044	.991
	Rash	-.376	.709			.596	.687	.170	2.772	.125	.135	.976
	Jaundice	-.202	1.283			.875	.817	.066	10.170	.101	.107	.980
	Haemorrhage	.361	.589			.540	1.435	.452	4.558	.074	.077	.985
	Pruritis	-1.398	1.106			.206	.247	.028	2.161	.014	.014	.997
	Lymphadenopathy	.693	.499			.165	2.000	.751	5.329	.099	.104	.981
	ConjunctSuffusion	-.819	.463			.077	.441	.178	1.094	.030	.030	.994
	ConjunctHaemorrhage	-3.082	2.118			.147	.046	.001	2.967	.125	.135	.976
	SoreThroat	-.822	.444			.064	.440	.184	1.050	.049	.050	.990
	Dysgeusia	.836	.327			.011	2.308	1.215	4.384	.021	.021	.996
	Headache	-.100	.387			.795	.904	.423	1.933	.033	.033	.993
	NeckStiffness	.251	1.274			.844	1.285	.105	15.716	.094	.100	.981
	Photophobia	-.164	.597			.783	.848	.263	2.742	.106	.113	.979
	Confusion	-2.099	2.035			.303	.123	.002	6.699	.105	.111	.979
	Hypotension	2.448	.605			.000	11.567	3.535	37.845	.033	.034	.993
	Myocarditis	2.005	1.882			.288	7.422	.184	300.158	.102	.108	.980
	ChestPain	.101	.434			.817	1.106	.472	2.592	.046	.047	.991
	Dyspnoea	-.384	.693			.580	.681	.175	2.651	.022	.022	.996
	Cough	-.376	.315			.233	.687	.370	1.274	.035	.035	.993
	Weightloss	-.489	1.087			.653	.613	.072	5.237	.163	.181	.968
	AbdominalPain	-.179	.414			.667	.837	.371	1.886	.064	.067	.987
	Nausea	.013	.317			.968	1.013	.545	1.884	.010	.010	.998
	Vomiting	.334	.290			.249	1.397	.792	2.465	.029	.029	.994
	Diarrhoea	.537	.372			.150	1.711	.822	3.562	.133	.144	.974
	Dysentery	1.460	1.447			.318	4.305	.236	78.500	.304	.384	.943
	Hepatomegaly	-.148	.488			.762	.862	.330	2.251	.095	.100	.981
	Splenomegaly	.911	.856			.289	2.486	.459	13.467	.161	.178	.969
	Oliguria	-1.772	1.436			.219	.170	.010	2.911	.186	.210	.964
	Dysuria	-.048	.416			.909	.954	.422	2.156	.052	.054	.990
	Myalgia	-.101	.381			.790	.904	.428	1.907	.056	.057	.989
	Arthralgia	-.090	.375			.811	.914	.438	1.909	.073	.076	.986
	ITU	-1.866	1.790			.300	.155	.004	5.452	.243	.288	.954
	Hct	.050	.040			.218	1.052	.968	1.142	.482	.770	.912
	Hb	.071	.076			.371	1.073	.908	1.269	.676	1.625	.881
	WCC	.016	.029			.577	1.017	.958	1.078	.363	.492	.932
	Neutrophils	-.028	.031			.389	.972	.908	1.042	.663	1.539	.883
	Lymphocytes	-.004	.006			.530	.996	.985	1.008	.195	.222	.962
	Plts	-.010	.002			.000	.990	.986	.995	.210	.242	.960
	CRP	-.029	.009			.005	.971	.952	.990	.535	.937	.903
	CPK	.000	.000			.208	1.000	1.000	1.000	.118	.127	.977
	Constant	-7.470	9.075			.411	.001	.000	31014.300	.071	.073	.986
	ReducedGCS	.944	2.108			.655	2.570	.040	166.740	.194	.221	.963

a. Variable(s) entered on step 1: Age, Male, PopulationDensity, Education, Income, PipedDrinkingWater, DelayedPresentation, AntibioticsPreAdmission, Smoking, Alcohol, Temperature, FeverIntermittent, Chills, Rigors, Wound, Flushing, Rash, Jaundice, Haemorrhage, Pruritis, Lymphadenopathy, ConjunctSuffusion, ConjunctHaemorrhage, SoreThroat, Dysgeusia, Headache, NeckStiffness, Photophobia, Confusion, Hypotension, Myocarditis, ChestPain, Dyspnoea, Cough, Weightloss, AbdominalPain, Nausea, Vomiting, Diarrhoea, Dysentery, Hepatomegaly, Splenomegaly, Oliguria, Dysuria, Myalgia, Arthralgia, ITU, Hct, Hb, WCC, Neutrophils, Lymphocytes, Plts, CRP, CPK.
b. Variable(s) entered on step 1: Age, Male, PopulationDensity, Education, Income, PipedDrinkingWater, DelayedPresentation, AntibioticsPreAdmission, Smoking, Alcohol, Temperature, FeverIntermittent, Chills, Rigors, Wound, Flushing, Rash, Jaundice, Haemorrhage, Pruritis, Lymphadenopathy, ConjunctSuffusion, ConjunctHaemorrhage, SoreThroat, Dysgeusia, Headache, NeckStiffness, Photophobia, Confusion, ReducedGCS, Hypotension, Myocarditis, ChestPain, Dyspnoea, Cough, Weightloss, AbdominalPain, Nausea, Vomiting, Diarrhoea, Dysentery, Hepatomegaly, Splenomegaly, Oliguria, Dysuria, Myalgia, Arthralgia, ITU, Hct, Hb, WCC, Neutrophils, Lymphocytes, Plts, CRP, CPK.

As shown in Table 3.3.1.1 this full adjusted model has a sensitivity of 78.5% and a specificity of 95.2% for identifying dengue cases with the original data set, but sensitivities of 60.6 – 63.5% and specificities of 95.2 – 96.1% with the imputed data sets. However, these figures are based on a probability estimate for dengue of 0.5 (50%), which may not be optimal for maximising both sensitivity & specificity. Comparing these figures for the original and imputed data sets indicates that there was bias towards improved sensitivity in the original data set.

Although sensitivity and specificity figures are important, they are not as useful clinically as positive predictive value (PPV) and negative predictive value (NPV). For the original data set the PPV was 62 / (62 + 11) = 84.9% and the NPV was 220 / (220 + 17) = 92.8%. For the first imputed data set the PPV was 87 / (87 + 18) = 82.9% and the NPV was 439 / (439 + 50) = 89.8%. The results for the other four imputed data sets were virtually identical and so are not reported.

The following nine variables (measured on admission) were identified from Table 3.3.1.2 as independent predictors of dengue with AORs that had a significance of $p < 0.1$:

- Antibiotics Pre Admission (0 = no, 1 = yes) → positive association
- Rigors (0 = no, 1 = yes) → positive association
- Flushing (0 = no, 1 = yes) → positive association
- Conjunctival Suffusion (0 = no, 1 = yes) → negative association
- Sore Throat (0 = no, 1 = yes) → negative association
- Dysgeusia (0 = no, 1 = yes) → positive association
- Hypotension (0 = no, 1 = yes) → positive association
- Platelets ($x10^9$/L) → negative association
- CRP (mg/L) → negative association

These variables were then used together in a repeat logistic regression to produce a parsimonious adjusted model for dengue as shown in Tables 3.3.1.3 and 3.3.1.4.

Table 3.3.1.3. Classification table for logistic regression of selected dengue predictors

Imputation Number		Observed		Predicted		
				Dengue Confirmed		
				No	Yes	Percentage Correct
Original data	Step 1	Dengue Confirmed	No	333	23	93.5
			Yes	53	71	57.3
		Overall Percentage				84.2
1	Step 1	Dengue Confirmed	No	430	27	94.1
			Yes	69	68	49.6
		Overall Percentage				83.8
2	Step 1	Dengue Confirmed	No	432	24	94.7
			Yes	73	64	46.7
		Overall Percentage				83.6
3	Step 1	Dengue Confirmed	No	431	25	94.5
			Yes	74	63	46.0
		Overall Percentage				83.3
4	Step 1	Dengue Confirmed	No	431	26	94.3
			Yes	73	64	46.7
		Overall Percentage				83.3
5	Step 1	Dengue Confirmed	No	429	27	94.1
			Yes	76	61	44.5
		Overall Percentage				82.6

a. The cut value is .500

Table 3.3.1.4. Logistic regression for parsimonious adjusted model for dengue

Variables in the Equation

Imputation Number			B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper	Fraction Missing Info.	Relative Increase Variance	Relative Efficiency
Original data	Step 1[a]	AntibioticsPreAdmission	.923	.319	8.375	1	.004	2.518	1.347	4.705			
		Rigors	.292	.275	1.128	1	.288	1.339	.781	2.294			
		Flushing	.941	.287	10.769	1	.001	2.562	1.461	4.493			
		ConjunctSuffusion	-.659	.442	2.223	1	.136	.517	.218	1.230			
		SoreThroat	-1.460	.423	11.894	1	.001	.232	.101	.532			
		Dysgeusia	1.169	.282	17.209	1	.000	3.220	1.853	5.595			
		Hypotension	2.331	.520	20.084	1	.000	10.285	3.711	28.504			
		Plts	-.010	.002	28.740	1	.000	.990	.987	.994			
		CRP	-.037	.007	32.517	1	.000	.964	.951	.976			
		Constant	.342	.416	.673	1	.412	1.407					
1	Step 1[a]	AntibioticsPreAdmission	.824	.294	7.850	1	.005	2.280	1.281	4.058			
		Rigors	.510	.250	4.152	1	.042	1.666	1.020	2.722			
		Flushing	.963	.264	13.309	1	.000	2.619	1.561	4.392			
		ConjunctSuffusion	-.780	.402	3.764	1	.052	.458	.208	1.008			
		SoreThroat	-.994	.366	7.403	1	.007	.370	.181	.757			
		Dysgeusia	1.069	.256	17.358	1	.000	2.911	1.761	4.813			
		Hypotension	1.922	.442	18.925	1	.000	6.834	2.875	16.246			
		Plts	-.008	.002	26.844	1	.000	.992	.989	.995			
		CRP	-.039	.006	36.717	1	.000	.962	.950	.974			
		Constant	-.138	.344	.162	1	.688	.871					
2	Step 1[a]	AntibioticsPreAdmission	.807	.284	8.067	1	.005	2.242	1.284	3.913			
		Rigors	.453	.247	3.371	1	.066	1.573	.970	2.551			
		Flushing	1.033	.261	15.604	1	.000	2.808	1.682	4.688			
		ConjunctSuffusion	-.813	.401	4.106	1	.043	.444	.202	.974			
		SoreThroat	-1.075	.363	8.794	1	.003	.341	.168	.695			
		Dysgeusia	1.044	.251	17.315	1	.000	2.840	1.737	4.642			
		Hypotension	2.001	.438	20.848	1	.000	7.398	3.134	17.465			
		Plts	-.009	.002	29.328	1	.000	.992	.988	.995			
		CRP	-.029	.005	32.555	1	.000	.972	.962	.981			
		Constant	-.109	.355	.094	1	.759	.897					
3	Step 1[a]	AntibioticsPreAdmission	.769	.289	7.096	1	.008	2.158	1.225	3.799			
		Rigors	.455	.247	3.391	1	.066	1.576	.971	2.558			
		Flushing	1.012	.261	15.005	1	.000	2.751	1.649	4.591			
		ConjunctSuffusion	-.789	.405	3.789	1	.052	.454	.205	1.005			
		SoreThroat	-.987	.365	7.298	1	.007	.373	.182	.763			
		Dysgeusia	.992	.252	15.472	1	.000	2.697	1.645	4.422			
		Hypotension	2.054	.453	20.599	1	.000	7.801	3.213	18.943			
		Plts	-.007	.002	20.752	1	.000	.993	.990	.996			
		CRP	-.037	.006	34.283	1	.000	.964	.952	.976			
		Constant	-.297	.345	.739	1	.390	.743					
4	Step 1[a]	AntibioticsPreAdmission	.757	.286	6.997	1	.008	2.131	1.217	3.734			
		Rigors	.471	.247	3.646	1	.056	1.601	.988	2.597			
		Flushing	1.068	.261	16.706	1	.000	2.909	1.743	4.854			
		ConjunctSuffusion	-.775	.399	3.782	1	.052	.461	.211	1.006			
		SoreThroat	-.950	.362	6.904	1	.009	.387	.190	.786			
		Dysgeusia	.990	.252	15.420	1	.000	2.690	1.642	4.408			
		Hypotension	2.030	.442	21.097	1	.000	7.613	3.202	18.102			
		Plts	-.007	.001	22.161	1	.000	.993	.990	.996			
		CRP	-.035	.006	34.318	1	.000	.965	.954	.977			
		Constant	-.306	.344	.794	1	.373	.736					
5	Step 1[a]	AntibioticsPreAdmission	.700	.282	6.140	1	.013	2.013	1.157	3.502			
		Rigors	.473	.246	3.710	1	.054	1.605	.992	2.597			
		Flushing	1.093	.260	17.702	1	.000	2.984	1.793	4.967			
		ConjunctSuffusion	-.812	.404	4.044	1	.044	.444	.201	.980			
		SoreThroat	-.917	.359	6.506	1	.011	.400	.198	.809			
		Dysgeusia	1.071	.249	18.484	1	.000	2.919	1.791	4.757			
		Hypotension	1.965	.436	20.269	1	.000	7.132	3.032	16.775			
		Plts	-.008	.001	27.382	1	.000	.992	.990	.995			
		CRP	-.027	.005	27.882	1	.000	.973	.963	.983			
		Constant	-.388	.329	1.389	1	.238	.678					
Pooled	Step 1[a]	AntibioticsPreAdmission	.771	.292			.008	2.163	1.220	3.834	.034	.034	.993
		Rigors	.472	.249			.057	1.604	.985	2.611	.010	.010	.998
		Flushing	1.034	.267			.000	2.811	1.664	4.749	.044	.045	.991
		ConjunctSuffusion	-.794	.403			.049	.452	.205	.995	.002	.002	1.000
		SoreThroat	-.985	.369			.008	.374	.181	.770	.032	.032	.994
		Dysgeusia	1.033	.256			.000	2.810	1.701	4.640	.030	.030	.994
		Hypotension	1.994	.446			.000	7.348	3.066	17.611	.017	.017	.997
		Plts	-.008	.002			.000	.992	.989	.996	.216	.251	.959
		CRP	-.033	.008			.001	.967	.951	.984	.536	.941	.903
		Constant	-.248	.367			.501	.781	.379	1.610	.133	.144	.974

a. Variable(s) entered on step 1: AntibioticsPreAdmission, Rigors, Flushing, ConjunctSuffusion, SoreThroat, Dysgeusia, Hypotension, Plts, CRP.

As shown in Table 3.3.1.3 this parsimonious adjusted model has a sensitivity of 57.3% and a specificity of 93.5% for identifying dengue cases with the original data set, but sensitivities of 44.5 – 49.6% and specificities of 94.1 – 94.7% with the imputed data sets. The pooled results in Table 3.3.1.4 show that one of the variables used (Rigors) no longer has an AOR with $P < 0.05$ in this model, but this was retained in the model as the intention at this stage was to identify all factors independently associated with dengue (and so a more relaxed P value of < 0.1 was adopted for this part of the analysis).

Continuous variables are more difficult to use in clinical prediction rules and so those for platelets and CRP on admission were considered for conversion into dichotomised variables by producing frequency and scatter plots and also by considering clinically relevant break-points. The resulting charts did not suggest any obvious break-point for dichotomisation of these variables and it would not be valid or useful to simply choose a mean or median value. However, from a clinical perspective the obvious break-point for platelets (which were often low in dengue) is the lower value in the normal range (150×10^9/L). The break-point for CRP is less obvious, but as shown in Section 3.2.15 a value of ≤12 mg/L would be expected in viral infections such as dengue. Therefore the following continuous variables were converted into dichotomised variables using the rules shown :

- Platelets $<150 \times 10^9$/L = Thrombocytopenia (0 = no, 1 = yes) → positive association
- CRP ≤12 mg/L = Low CRP (0 = no, 1 = yes) → positive association

The logistic regression was then repeated with these replacement variables to produce a parsimonious adjusted model using categorical variables only for dengue as shown in Tables 3.3.1.5 and 3.3.1.6.

Table 3.3.1.5. Classification table for logistic regression of selected dengue predictors (updated)

Classification Table[a]

Imputation Number		Observed		Predicted		
				Dengue Confirmed		Percentage Correct
				No	Yes	
Original data	Step 1	Dengue Confirmed	No	332	24	93.3
			Yes	59	65	52.4
		Overall Percentage				82.7
1	Step 1	Dengue Confirmed	No	429	28	93.9
			Yes	69	68	49.6
		Overall Percentage				83.7
2	Step 1	Dengue Confirmed	No	435	21	95.4
			Yes	74	63	46.0
		Overall Percentage				84.0
3	Step 1	Dengue Confirmed	No	428	28	93.9
			Yes	72	65	47.4
		Overall Percentage				83.1
4	Step 1	Dengue Confirmed	No	434	23	95.0
			Yes	72	65	47.4
		Overall Percentage				84.0
5	Step 1	Dengue Confirmed	No	432	24	94.7
			Yes	73	64	46.7
		Overall Percentage				83.6

a. The cut value is .500

Table 3.3.1.6. Logistic regression for parsimonious adjusted model for dengue (updated)

Variables in the Equation

Imputation Number			B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper	Fraction Missing Info.	Relative Increase Variance	Relative Efficiency
Original data	Step 1[a]	AntibioticsPreAdmission	.622	.303	4.222	1	.040	1.862	1.029	3.370			
		Rigors	.231	.267	.751	1	.386	1.260	.747	2.125			
		Flushing	1.179	.284	17.251	1	.000	3.252	1.864	5.672			
		ConjunctSuffusion	-.816	.442	3.407	1	.065	.442	.186	1.052			
		SoreThroat	-1.292	.403	10.299	1	.001	.275	.125	.605			
		Dysgeusia	1.206	.276	19.066	1	.000	3.339	1.944	5.737			
		Hypotension	2.025	.466	18.901	1	.000	7.579	3.041	18.886			
		Thrombocytopenia	1.293	.267	23.475	1	.000	3.645	2.160	6.150			
		LowCRP	1.765	.292	36.544	1	.000	5.839	3.295	10.347			
		Constant	-3.735	.389	92.152	1	.000	.024					
1	Step 1[a]	AntibioticsPreAdmission	.582	.281	4.280	1	.039	1.790	1.031	3.108			
		Rigors	.395	.245	2.605	1	.107	1.484	.919	2.396			
		Flushing	1.232	.263	21.906	1	.000	3.427	2.046	5.739			
		ConjunctSuffusion	-.913	.405	5.084	1	.024	.401	.181	.887			
		SoreThroat	-.893	.354	6.359	1	.012	.410	.205	.820			
		Dysgeusia	1.051	.252	17.344	1	.000	2.861	1.744	4.692			
		Hypotension	1.847	.422	19.145	1	.000	6.342	2.773	14.508			
		Thrombocytopenia	1.065	.243	19.228	1	.000	2.900	1.802	4.667			
		LowCRP	1.873	.272	47.342	1	.000	6.508	3.817	11.095			
		Constant	-3.882	.357	118.284	1	.000	.021					
2	Step 1[a]	AntibioticsPreAdmission	.518	.275	3.549	1	.060	1.679	.979	2.878			
		Rigors	.398	.243	2.688	1	.101	1.488	.925	2.394			
		Flushing	1.263	.262	23.252	1	.000	3.535	2.116	5.907			
		ConjunctSuffusion	-.877	.408	4.633	1	.031	.416	.187	.925			
		SoreThroat	-.931	.351	7.018	1	.008	.394	.198	.785			
		Dysgeusia	1.081	.250	18.721	1	.000	2.947	1.806	4.808			
		Hypotension	1.960	.426	21.159	1	.000	7.097	3.079	16.357			
		Thrombocytopenia	1.121	.242	21.471	1	.000	3.068	1.909	4.929			
		LowCRP	1.642	.262	39.333	1	.000	5.164	3.091	8.626			
		Constant	-3.721	.344	116.921	1	.000	.024					
3	Step 1[a]	AntibioticsPreAdmission	.543	.278	3.820	1	.051	1.721	.999	2.968			
		Rigors	.399	.242	2.709	1	.100	1.490	.927	2.396			
		Flushing	1.207	.261	21.324	1	.000	3.345	2.004	5.584			
		ConjunctSuffusion	-.935	.409	5.218	1	.022	.392	.176	.876			
		SoreThroat	-.913	.355	6.620	1	.010	.401	.200	.804			
		Dysgeusia	1.055	.250	17.811	1	.000	2.873	1.760	4.689			
		Hypotension	1.914	.425	20.241	1	.000	6.777	2.944	15.599			
		Thrombocytopenia	.967	.241	16.059	1	.000	2.630	1.639	4.221			
		LowCRP	1.713	.266	41.559	1	.000	5.547	3.295	9.339			
		Constant	-3.699	.343	116.487	1	.000	.025					
4	Step 1[a]	AntibioticsPreAdmission	.543	.276	3.866	1	.049	1.721	1.002	2.958			
		Rigors	.384	.242	2.525	1	.112	1.468	.914	2.357			
		Flushing	1.264	.260	23.576	1	.000	3.539	2.125	5.894			
		ConjunctSuffusion	-.966	.404	5.716	1	.017	.381	.173	.840			
		SoreThroat	-.856	.352	5.915	1	.015	.425	.213	.847			
		Dysgeusia	1.032	.249	17.174	1	.000	2.807	1.723	4.572			
		Hypotension	1.862	.415	20.156	1	.000	6.435	2.855	14.506			
		Thrombocytopenia	1.057	.241	19.290	1	.000	2.878	1.796	4.614			
		LowCRP	1.613	.261	38.083	1	.000	5.020	3.007	8.380			
		Constant	-3.663	.341	115.557	1	.000	.026					
5	Step 1[a]	AntibioticsPreAdmission	.481	.279	2.965	1	.085	1.617	.936	2.795			
		Rigors	.396	.244	2.650	1	.104	1.487	.922	2.396			
		Flushing	1.311	.264	24.686	1	.000	3.708	2.211	6.219			
		ConjunctSuffusion	-.925	.408	5.144	1	.023	.397	.178	.882			
		SoreThroat	-.873	.352	6.145	1	.013	.418	.210	.833			
		Dysgeusia	1.114	.251	19.648	1	.000	3.047	1.862	4.986			
		Hypotension	1.913	.425	20.305	1	.000	6.775	2.948	15.572			
		Thrombocytopenia	1.112	.240	21.464	1	.000	3.039	1.899	4.864			
		LowCRP	1.709	.266	41.242	1	.000	5.525	3.279	9.309			
		Constant	-3.815	.352	117.247	1	.000	.022					
Pooled	Step 1[a]	AntibioticsPreAdmission	.533	.281			.058	1.705	.983	2.957	.021	.022	.996
		Rigors	.394	.243			.105	1.483	.921	2.388	.001	.001	1.000
		Flushing	1.255	.266			.000	3.509	2.085	5.905	.026	.026	.995
		ConjunctSuffusion	-.923	.408			.024	.397	.178	.884	.008	.008	.998
		SoreThroat	-.893	.354			.012	.409	.204	.820	.009	.009	.998
		Dysgeusia	1.067	.253			.000	2.905	1.770	4.770	.019	.019	.996
		Hypotension	1.899	.425			.000	6.680	2.901	15.380	.014	.014	.997
		Thrombocytopenia	1.064	.250			.000	2.899	1.773	4.740	.074	.077	.985
		LowCRP	1.710	.288			.000	5.530	3.136	9.751	.156	.173	.970
		Constant	-3.756	.361			.000	.023	.012	.048	.077	.081	.985

a. Variable(s) entered on step 1: AntibioticsPreAdmission, Rigors, Flushing, ConjunctSuffusion, SoreThroat, Dysgeusia, Hypotension, Thrombocytopenia, LowCRP.

As shown in Table 3.3.1.5 this updated parsimonious adjusted model has a sensitivity of 52.4% and a specificity of 93.3% for identifying dengue cases with the original data set, but sensitivities of 46.0 – 49.6% and specificities of 93.9 – 95.4% with the imputed data sets. For the original data set the PPV was 65 / (65 + 24) = 73.0% and the NPV was 332 / (332 + 59) = 84.9%. For the first imputed data set the PPV was 68 / (68 + 28) = 70.8% and the NPV was 429 / (429 + 69) = 86.1%. The results for the other four imputed data sets were virtually identical and so are not reported. A summary of the diagnostic performance of the dengue prediction rule during its evolution is shown in Table 3.3.1.7.

Table 3.3.1.7. Diagnostic performance of the dengue prediction rule

	Full Model using Original Data	Full Model using Imputed Data	Final Model using Original Data	Final Model using Imputed Data
Sensitivity (95% CI)	78.5% (68.2–86.1%)	63.5% (55.2%–71.1%)	52.4% (43.7–61.0%)	49.6% (41.4–57.9%)
Specificity (95% CI)	95.2% (91.7–97.3%)	96.1% (93.9–97.5%)	93.3% (90.2–95.4%)	93.9% (91.3–95.7%)
PPV (95% CI)	84.9% (75.0–91.4%)	82.9% (74.5–88.9%)	73.0% (63.0–81.2%)	70.8% (61.1–79.0%)
NPV (95% CI)	92.8% (88.8–95.5%)	89.8% (86.8–92.2%)	84.9% (81.0–88.1%)	86.1% (82.8–88.9%)
LR+ (95% CI)	16.481 (9.153–29.677)	16.123 (10.074–25.803)	7.776 (5.103–11.848)	8.101 (5.449–12.044)
LR– (95% CI)	0.226 (0.148–0.345)	0.380 (0.304–0.474)	0.510 (0.423–0.615)	0.537 (0.454–0.635)

Using this parsimonious model with the original data set would enable more complete cases to be included (480 / 617 = 78% compared to 310 / 617 = 50% for the full model shown in Table 3.3.1.1). However, this would still leave 22% of cases with missing data and so the imputation approach remains appropriate. Two of the variables used (Antibiotics Pre Admission and Rigors) no longer have AORs with P

< 0.05 in this model, but these were retained in the model as the intention at this stage was to identify all factors independently associated with dengue (and so a more relaxed P value of < 0.1 was adopted for this part of the analysis).

Overall this updated model will be easier to use (since all of the continuous variables have been converted to dichotomous variables) and has equivalent accuracy compared to the original parsimonious model (in which Platelets and CRP were kept as a continuous variable). Therefore the updated model was chosen for construction of a scoring algorithm and further analysis using ROC curves.

The scoring algorithm was constructed as described previously (Section 2.3.1) using the LR coefficients (labelled "B") for the pooled imputation data set shown in Table 3.3.1.6. The scores for theoretical cases with the highest and lowest probabilities for having dengue are shown in Tables 3.3.1.8 and 3.3.1.9. The scores for the first confirmed dengue case (R006) and a confirmed non-dengue case (R269 who had chikungunya) are shown in Tables 3.3.1.10 and 3.3.1.11.

Table 3.3.1.8. Scoring algorithm for dengue showing highest probability

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Antibiotics pre-admission	0.533	**1**
Rigors	0.394	**1**
Flushing	1.255	**1**
Conjunctival suffusion	- 0.923	**0**
Sore throat	- 0.893	**0**
Dysgeusia	1.067	**1**
Hypotension	1.899	**1**
Thrombocytopenia	1.064	**1**
Low CRP	1.710	**1**
Constant	- 3.576	
Score	**4.166**	
Probability	**0.985**	

Table 3.3.1.9. Scoring algorithm for dengue showing lowest probability

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Antibiotics pre-admission	0.533	**0**
Rigors	0.394	**0**
Flushing	1.255	**0**
Conjunctival suffusion	- 0.923	**1**
Sore throat	- 0.893	**1**
Dysgeusia	1.067	**0**
Hypotension	1.899	**0**
Thrombocytopenia	1.064	**0**
Low CRP	1.710	**0**
Constant	- 3.576	
Score	**- 5.572**	
Probability	**0.004**	

Table 3.3.1.10. Scoring algorithm for a confirmed dengue case (R006)

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Antibiotics pre-admission	0.533	**0**
Rigors	0.394	**0**
Flushing	1.255	**1**
Conjunctival suffusion	- 0.923	**0**
Sore throat	- 0.893	**1**
Dysgeusia	1.067	**1**
Hypotension	1.899	**1**
Thrombocytopenia	1.064	**1**
Low CRP	1.710	**1**
Constant	- 3.576	
Score	**2.346**	
Probability	**0.913**	

Table 3.3.1.11. Scoring algorithm for a confirmed non-dengue case (R269)

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Antibiotics pre-admission	0.533	**0**
Rigors	0.394	**1**
Flushing	1.255	**0**
Conjunctival suffusion	- 0.923	**0**
Sore throat	- 0.893	**0**
Dysgeusia	1.067	**0**
Hypotension	1.899	**0**
Thrombocytopenia	1.064	**0**
Low CRP	1.710	**1**
Constant	- 3.576	
Score	**-1.652**	
Probability	**0.161**	

Although the maximum and minimum probabilities generated appear impressive, a cut-off for the probability needs to be identified that will give acceptable levels of both sensitivity and specificity for a clinical prediction rule. This is unlikely to be the intuitive level of 0.5 and so an appropriate cut-off was sought using ROC curve analysis. Using the LR coefficients produced (labelled as "B" values in the tables shown), probabilities were calculated and used to construct ROC curves for the original data and each of the 5 imputations. All ROC curves for the imputed datasets were similar and so one was chosen at random (Imputation 1) for further analysis. The ROC curves for the original data and this imputed data set are shown in Figures 3.3.1.1 and 3.3.1.2.

Figure 3.3.1.1. ROC curve for diagnosing dengue from nine selected variables using the original data set

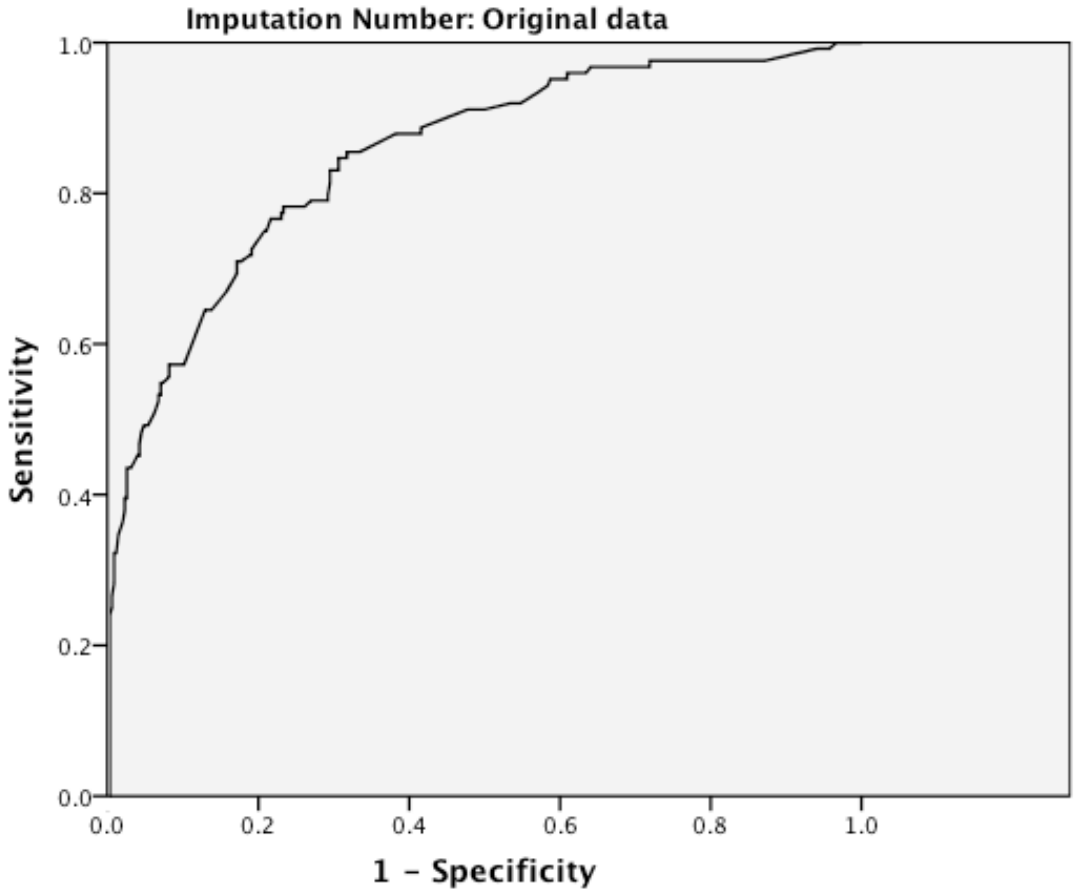
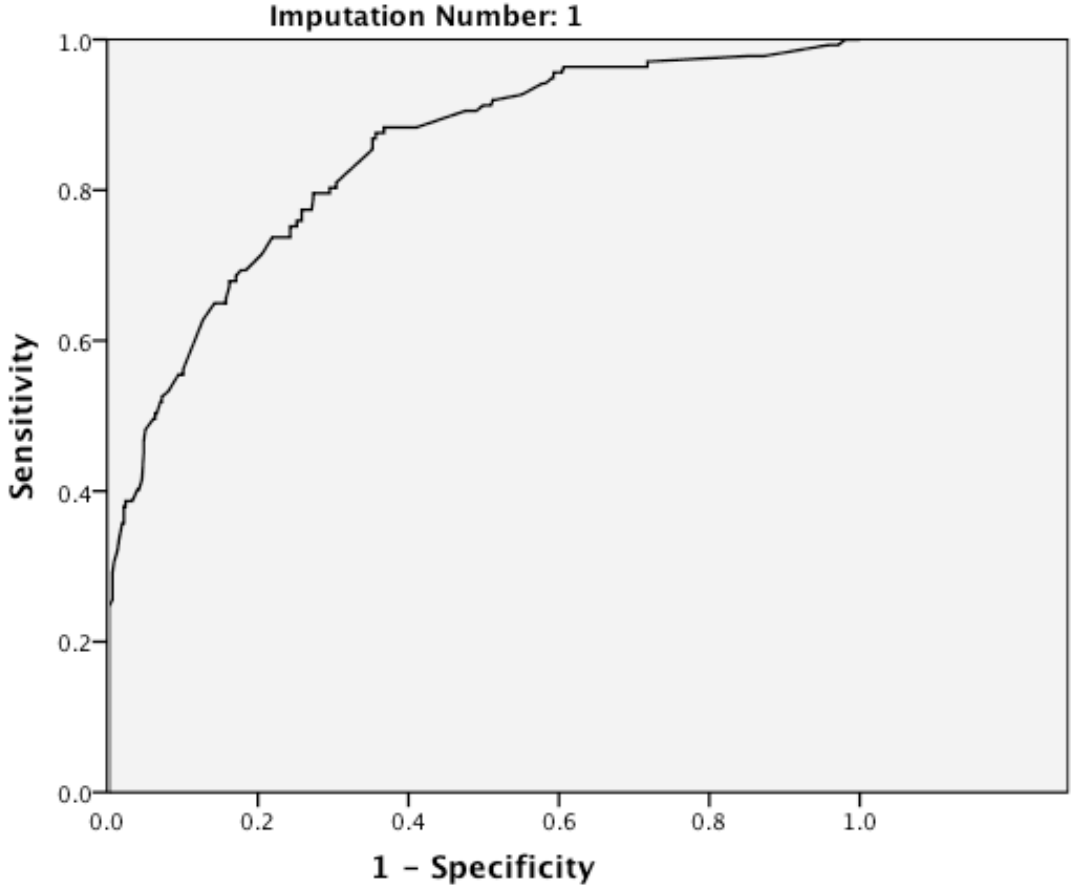


Figure 3.3.1.2. ROC curve for diagnosing dengue from nine selected variables using an imputed data set



Examination of the pivot table co-ordinates for these ROC curves shows that the following cut-offs will produce the following sensitivities and specificities :

Original Data :

Cut-Off = 0.0826511 : Sensitivity = 0.952 : Specificity = 1 – 0.587 = 0.413
Cut-Off = 0.5365252 : Sensitivity = 0.492 : Specificity = 1 – 0.048 = 0.952

Imputation 1 Data :

Cut-Off = 0.0679869 : Sensitivity = 0.956 : Specificity = 1 – 0.593 = 0.407
Cut-Off = 0.1169457 : Sensitivity = 0.905 : Specificity = 1 – 0.475 = 0.525
Cut-Off = 0.1831002 : Sensitivity = 0.803 : Specificity = 1 – 0.295 = 0.705

Cut-Off = 0.2786798 : Sensitivity = 0.715 : Specificity = 1 – 0.206 = 0.794
Cut-Off = 0.3643906 : Sensitivity = 0.628 : Specificity = 1 – 0.127 = 0.873
Cut-Off = 0.5064733 : Sensitivity = 0.482 : Specificity = 1 – 0.050 = 0.950

None of these probability cut-offs have both the sensitivity and specificity required for use in diagnosing dengue. However, the sensitivity and specificity values shown above indicate that the probability score for an individual patient based on this scoring system could offer some diagnostic information if taken in conjunction with other information available for that patient (but is not sufficiently accurate to form the basis of a diagnosis on its own).

3.3.2 Clinical Prediction Rule for the Diagnosis of Chikungunya

Variables recorded on admission that were potential predictors for a confirmed diagnosis of chikungunya were first subjected to univariate analysis. This included the use of Pearson's chi square test, Fisher's exact test and odds ratios for categorical variables and Student's independent samples t-test (with 2-tailed significance and Levene's test) for continuous variables. Using these tests the following variables were found to have unadjusted associations ($p < 0.05$) with chikungunya :

- Increasing population density
- Drinking water from a tap
- Early presentation to hospital
- Continuous fever pattern
- Flushing
- Lack of sore throat
- Headache
- Lack of cough
- Myalgia
- Lack of anaemia
- Decreasing CRP
- Decreasing education
- No antibiotics before admission
- Temperature
- Rigors
- Conjunctival suffusion
- Lack of dysgeusia
- Lack of dyspnoea
- Lack of abdominal pain
- Arthralgia
- Decreasing ESR

However, there was apparent overlap between these variables and so multivariate analysis using logistic regression was performed. Unfortunately, the use of complete case methodology led to 48.0% of all cases being excluded due to missing values and hence concerns regarding the representativeness of this analysis. Even when variables with >10% missing values were excluded, there were still >20% of all cases excluded from this type of analysis. Although these analyses of complete cases may have identified adjusted associations that were correct, there are also ethical issues regarding the omission of such a large amount of patient data.

Therefore a multiple imputation method was used with 5 iterations, which allowed the logistic regression to be repeated on the imputed data set (which then included 100% of cases). This identified some variables (occurrence of an eschar, conjunctival haemorrhage, myocarditis, haemoptysis, neurological signs or admission to ITU)

with no occurrences in the chikungunya cohort and so cases with these features were excluded from the remainder of this analysis (leaving 580 cases to be analysed) and so too were these variables themselves. These clinical features could be used to exclude chikungunya in this cohort of patients, but only occurred rarely (eschar in six cases, conjunctival haemorrhage in 4, myocarditis in four, haemoptysis in 16, neurological signs in one and admission to ITU in 11). Some of these features may have occurred with a larger sample size and would then have been included in the logistic regression.

The results of the final logistic regression on the imputed data sets are shown in Tables 3.3.2.1 and 3.3.2.2. In Table 3.3.2.1 the first row (labelled "Original data") shows that only 304 (52%) of the remaining 580 cases from the original data set would have been included in this analysis, compared to 100% of cases in the imputed datasets. In Table 3.3.2.2 the third column (labelled "S.E") shows that very few variables in the pooled imputation analysis had very high standard errors in this analysis, which indicated that the model was stable.

Table 3.3.2.1. Classification table for logistic regression of all chikungunya predictors

				Predicted		
				Chikungunya Confirmed		
Imputation Number		Observed		No	Yes	Percentage Correct
Original data	Step 1	Chikungunya Confirmed	No	243	6	97.6
			Yes	13	42	76.4
		Overall Percentage				93.8
1	Step 1	Chikungunya Confirmed	No	457	20	95.8
			Yes	47	56	54.4
		Overall Percentage				88.4
2	Step 1	Chikungunya Confirmed	No	458	19	96.0
			Yes	44	58	56.9
		Overall Percentage				89.1
3	Step 1	Chikungunya Confirmed	No	460	18	96.2
			Yes	48	54	52.9
		Overall Percentage				88.6
4	Step 1	Chikungunya Confirmed	No	458	20	95.8
			Yes	52	50	49.0
		Overall Percentage				87.6
5	Step 1	Chikungunya Confirmed	No	461	17	96.4
			Yes	55	47	46.1
		Overall Percentage				87.6

a. The cut value is .500

Table 3.3.2.2. Logistic regression for full adjusted model for chikungunya

Imputation Number		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper	Fraction Missing Info.	Relative Increase Variance	Relative Efficiency
Pooled	Step 1[a,b] Age	.020	.012			.086	1.020	.997	1.044	.024	.024	.995
	Male	.919	.480			.058	2.508	.970	6.484	.191	.217	.963
	PopulationDensity	.000	.000			.300	1.000	1.000	1.001	.154	.170	.970
	Education	.000	.051			.994	1.000	.904	1.106	.115	.123	.978
	Income	.000	.000			.330	1.000	1.000	1.000	.203	.233	.961
	PipedDrinkingWater	.737	.360			.042	2.090	1.026	4.257	.180	.203	.965
	DelayedPresentation	-.291	.090			.002	.747	.625	.894	.249	.298	.953
	AntibioticsPreAdmission	-.140	.446			.753	.869	.362	2.086	.056	.058	.989
	Smoking	.072	.456			.875	1.074	.440	2.625	.036	.037	.993
	Alcohol	-.331	.391			.397	.718	.334	1.547	.083	.088	.984
	Temperature	.505	.242			.037	1.658	1.031	2.665	.031	.032	.994
	FeverIntermittent	.487	.314			.121	1.628	.879	3.014	.022	.023	.996
	Chills	-.183	.696			.793	.833	.212	3.274	.098	.104	.981
	Rigors	.473	.335			.158	1.605	.832	3.098	.041	.042	.992
	Wound	-.161	.609			.792	.851	.258	2.812	.053	.054	.990
	Flushing	.340	.377			.367	1.405	.670	2.945	.102	.108	.980
	Rash	-.287	.849			.735	.750	.142	3.959	.016	.016	.997
	Jaundice	1.142	1.104			.301	3.133	.359	27.343	.054	.055	.989
	Haemorrhage	-.087	.708			.902	.917	.228	3.680	.071	.074	.986
	Pruritis	-.350	1.744			.841	.705	.023	21.513	.016	.016	.997
	Lymphadenopathy	-.116	.561			.837	.891	.297	2.674	.014	.014	.997
	ConjunctSuffusion	.399	.463			.391	1.490	.596	3.726	.192	.218	.963
	SoreThroat	-.890	.582			.126	.411	.131	1.284	.030	.030	.994
	Dysgeusia	-1.430	.380			.000	.239	.114	.504	.026	.026	.995
	Headache	1.067	.451			.018	2.907	1.200	7.039	.033	.033	.993
	NeckStiffness	-14.092	9722.104			.999	.000	.000	Infinity	.000	.000	1.000
	Photophobia	.518	.612			.397	1.679	.506	5.573	.045	.046	.991
	Confusion	1.029	1.617			.525	2.798	.116	67.641	.133	.144	.974
	Hypotension	.099	.680			.885	1.104	.291	4.188	.017	.017	.997
	ChestPain	.503	.467			.282	1.654	.661	4.139	.083	.087	.984
	Dyspnoea	-1.857	1.220			.128	.156	.014	1.706	.007	.007	.999
	Cough	-.050	.341			.885	.952	.488	1.857	.005	.005	.999
	Weightloss	1.013	1.312			.440	2.754	.211	36.021	.019	.020	.996
	AbdominalPain	-.779	.527			.139	.459	.163	1.288	.005	.005	.999
	Nausea	.089	.379			.815	1.093	.520	2.297	.015	.015	.997
	Vomiting	-.086	.307			.780	.918	.503	1.674	.008	.008	.998
	Diarrhoea	-.611	.488			.211	.543	.208	1.418	.133	.145	.974
	Dysentery	-3.025	8709.435			1.000	.049	.000	Infinity	.000	.000	1.000
	Hepatomegaly	.528	.502			.293	1.695	.634	4.533	.028	.028	.994
	Splenomegaly	.157	1.612			.923	1.170	.046	29.946	.320	.412	.940
	Oliguria	.758	1.292			.559	2.134	.165	27.603	.201	.231	.961
	Dysuria	.425	.455			.350	1.530	.627	3.735	.035	.036	.993
	Myalgia	-.180	.414			.665	.836	.371	1.884	.062	.064	.988
	Arthralgia	1.888	.448			.000	6.605	2.744	15.901	.054	.056	.989
	Hct	-.021	.042			.624	.980	.898	1.069	.511	.858	.907
	Hb	-.011	.040			.780	.989	.911	1.073	.412	.593	.924
	WCC	-.054	.057			.362	.947	.837	1.072	.614	1.265	.891
	Neutrophils	-.021	.023			.377	.979	.933	1.028	.515	.869	.907
	Lymphocytes	.004	.012			.743	1.004	.979	1.030	.495	.807	.910
	Plts	.001	.002			.667	1.001	.996	1.005	.135	.147	.974
	CRP	-.025	.010			.032	.975	.954	.997	.601	1.201	.893
	CPK	.000	.000			.467	1.000	.999	1.000	.612	1.253	.891
	Constant	-22.001	9.797			.025	.000	.000	.062	.050	.051	.990
	ReducedGCS	-15.354	8138.445			.999	.000	.000	Infinity	.000	.000	1.000

a. Variable(s) entered on step 1: Age, Male, PopulationDensity, Education, Income, PipedDrinkingWater, DelayedPresentation, AntibioticsPreAdmission, Smoking, Alcohol, Temperature, FeverIntermittent, Chills, Rigors, Wound, Flushing, Rash, Jaundice, Haemorrhage, Pruritis, Lymphadenopathy, ConjunctSuffusion, SoreThroat, Dysgeusia, Headache, NeckStiffness, Photophobia, Confusion, Hypotension, ChestPain, Dyspnoea, Cough, Weightloss, AbdominalPain, Nausea, Vomiting, Diarrhoea, Dysentery, Hepatomegaly, Splenomegaly, Oliguria, Dysuria, Myalgia, Arthralgia, Hct, Hb, WCC, Neutrophils, Lymphocytes, Plts, CRP, CPK.
b. Variable(s) entered on step 1: Age, Male, PopulationDensity, Education, Income, PipedDrinkingWater, DelayedPresentation, AntibioticsPreAdmission, Smoking, Alcohol, Temperature, FeverIntermittent, Chills, Rigors, Wound, Flushing, Rash, Jaundice, Haemorrhage, Pruritis, Lymphadenopathy, ConjunctSuffusion, SoreThroat, Dysgeusia, Headache, NeckStiffness, Photophobia, Confusion, ReducedGCS, Hypotension, ChestPain, Dyspnoea, Cough, Weightloss, AbdominalPain, Nausea, Vomiting, Diarrhoea, Dysentery, Hepatomegaly, Splenomegaly, Oliguria, Dysuria, Myalgia, Arthralgia, Hct, Hb, WCC, Neutrophils, Lymphocytes, Plts, CRP, CPK.

As shown in Table 3.3.2.1 this full adjusted model has a sensitivity of 76.4% and a specificity of 97.6% for identifying chikungunya cases with the original data set, but sensitivities of 46.1 – 56.9% and specificities of 95.8 – 96.4% with the imputed data sets. However, these figures are based on a probability estimate for chikungunya of 0.5 (50%), which may not be optimal for maximising both sensitivity & specificity. Comparing these figures for the original and imputed data sets indicates that there was bias towards improved sensitivity in the original data set.

Although sensitivity and specificity figures are important, they are not as useful clinically as positive predictive value (PPV) and negative predictive value (NPV). For the original data set the PPV was 42 / (42 + 6) = 87.5% and the NPV was 243 / (243 + 13) = 94.9%. For the first imputed data set the PPV was 56 / (56 + 20) = 73.7% and the NPV was 457 / (457 + 47) = 90.7%. The results for the other four imputed data sets were virtually identical and so are not reported.

The following nine variables (measured on admission) were identified from Table 3.3.2.2 as independent predictors of chikungunya with AORs that had a significance of $P < 0.1$:

- Age (years) → positive association
- Male (0 = no, 1 = yes) → positive association
- Piped Drinking Water (0 = no, 1 = yes) → positive association
- Delayed Presentation (days from onset to admission) → negative association
- Temperature (°C) → positive association
- Dysgeusia (0 = no, 1 = yes) → negative association
- Headache (0 = no, 1 = yes) → positive association
- Arthralgia (0 = no, 1 = yes) → positive association
- CRP (mg/L) → negative association

These variables were then used together in a repeat logistic regression that produced the results shown in Table 3.2.2.3 and 3.2.2.4.

Table 3.3.2.3. Classification table for logistic regression of selected chikungunya predictors

Classification Table[a]

Imputation Number		Observed		Predicted		
				Chikungunya Confirmed		
				No	Yes	Percentage Correct
Original data	Step 1	Chikungunya Confirmed	No	401	21	95.0
			Yes	53	37	41.1
		Overall Percentage				85.5
1	Step 1	Chikungunya Confirmed	No	454	23	95.2
			Yes	60	43	41.7
		Overall Percentage				85.7
2	Step 1	Chikungunya Confirmed	No	452	25	94.8
			Yes	59	43	42.2
		Overall Percentage				85.5
3	Step 1	Chikungunya Confirmed	No	452	26	94.6
			Yes	63	39	38.2
		Overall Percentage				84.7
4	Step 1	Chikungunya Confirmed	No	454	24	95.0
			Yes	62	40	39.2
		Overall Percentage				85.2
5	Step 1	Chikungunya Confirmed	No	456	22	95.4
			Yes	67	35	34.3
		Overall Percentage				84.7

a. The cut value is .500

Table 3.3.2.4. Logistic regression for parsimonious adjusted model for chikungunya

Variables in the Equation

Imputation Number			B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper	Fraction Missing Info.	Relative Increase Variance	Relative Efficiency
Original data	Step 1[a]	Age	.019	.010	3.683	1	.055	1.019	1.000	1.039			
		Male	.574	.328	3.058	1	.080	1.776	.933	3.380			
		PipedDrinkingWater	.985	.286	11.850	1	.001	2.678	1.528	4.691			
		DelayedPresentation	-.252	.068	13.722	1	.000	.777	.680	.888			
		Temperature	.470	.218	4.667	1	.031	1.601	1.045	2.452			
		Dysgeusia	-2.034	.353	33.228	1	.000	.131	.065	.261			
		Headache	1.221	.401	9.275	1	.002	3.389	1.545	7.434			
		Arthralgia	1.833	.356	26.444	1	.000	6.254	3.110	12.577			
		CRP	-.027	.006	17.678	1	.000	.973	.961	.986			
		Constant	-21.665	8.578	6.379	1	.012	.000					
1	Step 1[a]	Age	.023	.009	6.194	1	.013	1.023	1.005	1.041			
		Male	.609	.303	4.032	1	.045	1.838	1.015	3.331			
		PipedDrinkingWater	.886	.263	11.381	1	.001	2.425	1.449	4.056			
		DelayedPresentation	-.264	.064	16.822	1	.000	.768	.676	.871			
		Temperature	.476	.196	5.886	1	.015	1.609	1.096	2.364			
		Dysgeusia	-1.787	.319	31.439	1	.000	.167	.090	.313			
		Headache	1.213	.382	10.098	1	.001	3.364	1.592	7.110			
		Arthralgia	1.798	.324	30.729	1	.000	6.039	3.198	11.404			
		CRP	-.026	.006	19.915	1	.000	.974	.963	.985			
		Constant	-21.992	7.715	8.126	1	.004	.000					
2	Step 1[a]	Age	.019	.009	4.130	1	.042	1.019	1.001	1.037			
		Male	.505	.302	2.792	1	.095	1.658	.916	2.999			
		PipedDrinkingWater	.866	.266	10.637	1	.001	2.377	1.413	4.000			
		DelayedPresentation	-.282	.067	17.859	1	.000	.754	.662	.860			
		Temperature	.484	.201	5.796	1	.016	1.623	1.094	2.407			
		Dysgeusia	-1.851	.323	32.925	1	.000	.157	.083	.296			
		Headache	1.218	.381	10.218	1	.001	3.381	1.602	7.134			
		Arthralgia	1.855	.333	31.022	1	.000	6.395	3.329	12.285			
		CRP	-.028	.006	23.709	1	.000	.972	.962	.983			
		Constant	-22.004	7.906	7.747	1	.005	.000					
3	Step 1[a]	Age	.022	.009	5.581	1	.018	1.022	1.004	1.040			
		Male	.630	.304	4.297	1	.038	1.878	1.035	3.409			
		PipedDrinkingWater	.867	.264	10.809	1	.001	2.380	1.419	3.991			
		DelayedPresentation	-.258	.066	15.504	1	.000	.772	.679	.878			
		Temperature	.455	.197	5.353	1	.021	1.576	1.072	2.316			
		Dysgeusia	-1.786	.317	31.755	1	.000	.168	.090	.312			
		Headache	1.171	.379	9.547	1	.002	3.226	1.535	6.781			
		Arthralgia	1.808	.329	30.103	1	.000	6.096	3.196	11.628			
		CRP	-.026	.006	19.568	1	.000	.974	.963	.985			
		Constant	-21.137	7.735	7.467	1	.006	.000					
4	Step 1[a]	Age	.019	.009	4.365	1	.037	1.019	1.001	1.037			
		Male	.559	.301	3.461	1	.063	1.749	.970	3.152			
		PipedDrinkingWater	.846	.263	10.359	1	.001	2.330	1.392	3.899			
		DelayedPresentation	-.251	.065	15.140	1	.000	.778	.685	.883			
		Temperature	.459	.198	5.348	1	.021	1.582	1.072	2.334			
		Dysgeusia	-1.786	.317	31.831	1	.000	.168	.090	.312			
		Headache	1.147	.377	9.261	1	.002	3.149	1.504	6.593			
		Arthralgia	1.839	.327	31.520	1	.000	6.287	3.309	11.946			
		CRP	-.027	.006	20.364	1	.000	.973	.962	.985			
		Constant	-21.140	7.793	7.359	1	.007	.000					
5	Step 1[a]	Age	.018	.009	3.896	1	.048	1.018	1.000	1.036			
		Male	.571	.297	3.697	1	.055	1.770	.989	3.167			
		PipedDrinkingWater	.776	.257	9.125	1	.003	2.174	1.313	3.598			
		DelayedPresentation	-.244	.063	15.111	1	.000	.784	.693	.886			
		Temperature	.459	.194	5.586	1	.018	1.582	1.081	2.314			
		Dysgeusia	-1.679	.311	29.125	1	.000	.187	.101	.343			
		Headache	1.137	.370	9.435	1	.002	3.119	1.509	6.444			
		Arthralgia	1.809	.323	31.326	1	.000	6.106	3.240	11.506			
		CRP	-.018	.005	13.335	1	.000	.982	.973	.992			
		Constant	-21.243	7.619	7.775	1	.005	.000					
Pooled	Step 1[a]	Age	.020	.009			.034	1.020	1.002	1.039	.062	.065	.988
		Male	.575	.306			.060	1.777	.975	3.238	.030	.031	.994
		PipedDrinkingWater	.848	.266			.001	2.335	1.385	3.938	.031	.032	.994
		DelayedPresentation	-.260	.067			.000	.771	.676	.879	.057	.059	.989
		Temperature	.466	.198			.018	1.594	1.082	2.349	.005	.005	.999
		Dysgeusia	-1.778	.324			.000	.169	.089	.319	.045	.046	.991
		Headache	1.177	.380			.002	3.246	1.541	6.837	.011	.012	.998
		Arthralgia	1.822	.329			.000	6.183	3.247	11.774	.006	.007	.999
		CRP	-.025	.007			.002	.975	.961	.990	.429	.632	.921
		Constant	-21.503	7.770			.006	.000	.000	.002	.004	.004	.999

a. Variable(s) entered on step 1: Age, Male, PipedDrinkingWater, DelayedPresentation, Temperature, Dysgeusia, Headache, Arthralgia, CRP.

As shown in Table 3.3.2.3 this parsimonious adjusted model has a sensitivity of 41.1% and a specificity of 95.0% for identifying chikungunya cases with the original data set, but sensitivities of 34.3 – 42.2% and specificities of 94.8 – 95.4% with the imputed data sets. The pooled results in Table 3.3.2.4 show that one of the variables used (Male) no longer has an AOR with $P < 0.05$ in this model, but this was retained in the model as the intention at this stage was to identify all factors independently associated with dengue (and so a more relaxed P value of < 0.1 was adopted for this part of the analysis).

Continuous variables are more difficult to use in clinical prediction rules and so those for age, delayed presentation, temperature and CRP on admission were considered for conversion into dichotomised variables by producing frequency and scatter plots and also by considering clinically relevant break-points. The resulting charts did not suggest any obvious break-point for dichotomisation of these variables and it would not be valid or useful to simply choose a mean or median value. Also from a clinical perspective there are no obvious break-points for age, delayed presentation and temperature. A break-point for CRP is more valid because (as shown in Section 3.2.15) a value of ≤12 mg/L would be expected in viral infections such as dengue. Therefore this was the only continuous variable to be converted into a dichotomised variables using the rule shown :

- CRP ≤12 mg/L = Low CRP (0 = no, 1 = yes) → positive association

The logistic regression was then repeated with this replacement variable to produce a parsimonious adjusted model using mostly categorical variables and the other three continuous variables for chikungunya as shown in Tables 3.3.2.5 and 3.3.2.6.

Table 3.3.2.5. Classification table for logistic regression of selected chikungunya predictors (updated)

Classification Table[a]

Imputation Number		Observed		Predicted		
				Chikungunya Confirmed		
				No	Yes	Percentage Correct
Original data	Step 1	Chikungunya Confirmed	No	402	20	95.3
			Yes	58	32	35.6
		Overall Percentage				84.8
1	Step 1	Chikungunya Confirmed	No	453	24	95.0
			Yes	67	36	35.0
		Overall Percentage				84.3
2	Step 1	Chikungunya Confirmed	No	454	23	95.2
			Yes	68	34	33.3
		Overall Percentage				84.3
3	Step 1	Chikungunya Confirmed	No	451	27	94.4
			Yes	69	33	32.4
		Overall Percentage				83.4
4	Step 1	Chikungunya Confirmed	No	455	23	95.2
			Yes	68	34	33.3
		Overall Percentage				84.3
5	Step 1	Chikungunya Confirmed	No	456	22	95.4
			Yes	72	30	29.4
		Overall Percentage				83.8

a. The cut value is .500

Table 3.3.2.6. Logistic regression for parsimonious adjusted model for chikungunya (updated)

Variables in the Equation

Imputation Number			B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	95% C.I.for EXP(B) Upper	Fraction Missing Info.	Relative Increase Variance	Relative Efficiency
Original data	Step 1[a]	Age	.015	.010	2.542	1	.111	1.015	.996	1.035			
		Male	.473	.322	2.159	1	.142	1.604	.854	3.014			
		PipedDrinkingWater	.903	.278	10.541	1	.001	2.468	1.431	4.258			
		DelayedPresentation	-.271	.067	16.186	1	.000	.762	.668	.870			
		Temperature	.404	.212	3.635	1	.057	1.499	.989	2.271			
		Dysgeusia	-1.972	.350	31.761	1	.000	.139	.070	.276			
		Headache	1.162	.391	8.844	1	.003	3.197	1.486	6.877			
		Arthralgia	1.812	.354	26.119	1	.000	6.120	3.055	12.260			
		LowCRP	1.054	.300	12.313	1	.000	2.870	1.593	5.170			
		Constant	-19.978	8.362	5.708	1	.017	.000					
1	Step 1[a]	Age	.020	.009	4.947	1	.026	1.020	1.002	1.038			
		Male	.479	.297	2.601	1	.107	1.615	.902	2.893			
		PipedDrinkingWater	.824	.257	10.236	1	.001	2.279	1.376	3.774			
		DelayedPresentation	-.277	.064	18.802	1	.000	.758	.669	.859			
		Temperature	.427	.193	4.915	1	.027	1.533	1.051	2.237			
		Dysgeusia	-1.768	.317	31.099	1	.000	.171	.092	.318			
		Headache	1.172	.374	9.842	1	.002	3.229	1.552	6.716			
		Arthralgia	1.793	.325	30.533	1	.000	6.009	3.181	11.352			
		LowCRP	1.121	.279	16.156	1	.000	3.067	1.776	5.297			
		Constant	-21.063	7.597	7.687	1	.006	.000					
2	Step 1[a]	Age	.015	.009	3.046	1	.081	1.015	.998	1.033			
		Male	.419	.295	2.020	1	.155	1.521	.853	2.711			
		PipedDrinkingWater	.788	.258	9.334	1	.002	2.199	1.326	3.646			
		DelayedPresentation	-.291	.065	19.781	1	.000	.747	.657	.850			
		Temperature	.439	.195	5.058	1	.025	1.551	1.058	2.275			
		Dysgeusia	-1.777	.318	31.166	1	.000	.169	.091	.316			
		Headache	1.128	.371	9.247	1	.002	3.088	1.493	6.387			
		Arthralgia	1.838	.328	31.310	1	.000	6.281	3.300	11.956			
		LowCRP	1.096	.277	15.665	1	.000	2.991	1.739	5.146			
		Constant	-21.241	7.687	7.636	1	.006	.000					
3	Step 1[a]	Age	.017	.009	3.612	1	.057	1.017	.999	1.034			
		Male	.510	.297	2.961	1	.085	1.666	.932	2.980			
		PipedDrinkingWater	.780	.256	9.294	1	.002	2.181	1.321	3.599			
		DelayedPresentation	-.275	.065	18.213	1	.000	.759	.669	.862			
		Temperature	.386	.191	4.091	1	.043	1.471	1.012	2.139			
		Dysgeusia	-1.696	.312	29.529	1	.000	.183	.099	.338			
		Headache	1.107	.367	9.092	1	.003	3.025	1.473	6.211			
		Arthralgia	1.803	.325	30.824	1	.000	6.067	3.211	11.466			
		LowCRP	.854	.271	9.940	1	.002	2.349	1.381	3.995			
		Constant	-19.171	7.515	6.507	1	.011	.000					
4	Step 1[a]	Age	.017	.009	3.709	1	.054	1.017	1.000	1.035			
		Male	.490	.295	2.749	1	.097	1.632	.915	2.911			
		PipedDrinkingWater	.762	.257	8.813	1	.003	2.142	1.295	3.541			
		DelayedPresentation	-.261	.063	17.022	1	.000	.770	.680	.872			
		Temperature	.429	.195	4.840	1	.028	1.536	1.048	2.252			
		Dysgeusia	-1.732	.313	30.639	1	.000	.177	.096	.327			
		Headache	1.113	.370	9.074	1	.003	3.044	1.475	6.282			
		Arthralgia	1.775	.326	29.699	1	.000	5.901	3.116	11.173			
		LowCRP	1.104	.280	15.557	1	.000	3.016	1.743	5.221			
		Constant	-21.012	7.691	7.464	1	.006	.000					
5	Step 1[a]	Age	.015	.009	3.032	1	.082	1.015	.998	1.033			
		Male	.491	.293	2.809	1	.094	1.633	.920	2.899			
		PipedDrinkingWater	.735	.253	8.413	1	.004	2.086	1.269	3.428			
		DelayedPresentation	-.256	.062	16.880	1	.000	.774	.685	.875			
		Temperature	.409	.190	4.612	1	.032	1.505	1.036	2.185			
		Dysgeusia	-1.657	.310	28.662	1	.000	.191	.104	.350			
		Headache	1.077	.364	8.754	1	.003	2.936	1.439	5.994			
		Arthralgia	1.804	.323	31.267	1	.000	6.075	3.228	11.434			
		LowCRP	.793	.270	8.613	1	.003	2.210	1.301	3.753			
		Constant	-19.960	7.485	7.110	1	.008	.000					
Pooled	Step 1[a]	Age	.017	.009			.063	1.017	.999	1.035	.049	.050	.990
		Male	.478	.298			.109	1.613	.900	2.891	.016	.017	.997
		PipedDrinkingWater	.778	.259			.003	2.176	1.311	3.614	.019	.020	.996
		DelayedPresentation	-.272	.066			.000	.762	.670	.867	.055	.057	.989
		Temperature	.418	.194			.031	1.519	1.038	2.223	.014	.014	.997
		Dysgeusia	-1.726	.319			.000	.178	.095	.332	.030	.030	.994
		Headache	1.119	.371			.003	3.063	1.480	6.338	.011	.011	.998
		Arthralgia	1.803	.326			.000	6.066	3.201	11.495	.006	.006	.999
		LowCRP	.994	.325			.004	2.701	1.407	5.182	.307	.390	.942
		Constant	-20.489	7.658			.007	.000	.000	.004	.016	.017	.997

a. Variable(s) entered on step 1: Age, Male, PipedDrinkingWater, DelayedPresentation, Temperature, Dysgeusia, Headache, Arthralgia, LowCRP.

As shown in Table 3.3.2.5 this updated parsimonious adjusted model has a sensitivity of 35.6% and a specificity of 95.3% for identifying chikungunya cases with the original data set, but sensitivities of 29.4 – 35.0% and specificities of 94.4 – 95.4% with the imputed data sets. For the original data set the PPV was 32 / (32 + 20) = 61.5% and the NPV was 402 / (402 + 58) = 87.4%. For the first imputed data set the PPV was 36 / (36 + 24) = 60.0% and the NPV was 453 / (453 + 67) = 87.1%. The results for the other four imputed data sets were virtually identical and so are not reported. A summary of the diagnostic performance of the chikungunya prediction rule during its evolution is shown in Table 3.3.2.7.

Table 3.3.2.7. Diagnostic performance of the chikungunya prediction rule

	Full Model using Original Data	Full Model using Imputed Data	Final Model using Original Data	Final Model using Imputed Data
Sensitivity (95% CI)	76.4% (63.7–85.6%)	54.4% (44.8–63.7%)	35.6% (26.4–45.8%)	35.0% (26.4–44.5%)
Specificity (95% CI)	97.6% (94.8–98.9%)	95.8% (93.6–97.3%)	95.3% (92.8–96.9%)	95.0% (92.6–96.6%)
PPV (95% CI)	87.5% (75.3–94.1%)	73.7% (62.8–82.3%)	61.5% (48.0–73.5%)	60.0% (47.4–71.4%)
NPV (95% CI)	94.9% (91.5–97.0%)	90.7% (87.8–92.9%)	87.4% (84.0–90.1%)	87.1% (84.0–89.7%)
LR+ (95% CI)	31.691 (14.183–70.813)	12.967 (8.153–20.624)	7.502 (4.504–12.496)	6.947 (4.339–11.121)
LR– (95% CI)	0.242 (0.151–0.390)	0.476 (0.385–0.589)	0.677 (0.579–0.790)	0.685 (0.594–0.790)

Using this parsimonious model with the original data set would enable more complete cases to be included (512 / 617 = 83% compared to 304 / 617 = 49% for the full model shown in Table 3.3.2.1). However, this would still leave 17% of cases with missing data and so the imputation approach remains appropriate. Two of the variables used (Age and Male) no longer have AORs with $P < 0.05$ in this model, but

these were retained in the model as the intention at this stage was to identify all factors independently associated with dengue (and so a more relaxed P value of < 0.1 was adopted for this part of the analysis).

Overall this updated model will be easier to use (since one of the continuous variables has been converted to a dichotomous variable), but has decreased accuracy compared to the original parsimonious model (in which CRP was kept as a continuous variable). Therefore the updated model was chosen for construction of a scoring algorithm and further analysis using ROC curves.

The scoring algorithm was constructed as described previously (Section 2.3.1) using the LR coefficients (labelled "B") for the pooled imputation data set shown in Table 3.3.2.6. The scores for theoretical cases with the highest and lowest probabilities for having dengue are shown in Tables 3.3.2.8 and 3.3.2.9. The scores for the first confirmed chikungunya case (R269) and a confirmed non-chikungunya case (R006 who had dengue) are shown in Tables 3.3.2.10 and 3.3.2.11.

Table 3.3.2.8. Scoring algorithm for chikungunya showing highest probability

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Age (years)	0.017	**90**
Male	0.478	**1**
Piped drinking water	0.778	**1**
Delayed presentation (days)	- 0.272	**0**
Temperature (°C)	0.418	**41.0**
Dysgeusia	- 1.726	**0**
Headache	1.119	**1**
Arthralgia	1.803	**1**
Low CRP	0.994	**1**
Constant	- 20.489	
Score	**3.351**	
Probability	**0.966**	

Table 3.3.2.9. Scoring algorithm for chikungunya showing lowest probability

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Age (years)	0.017	**16**
Male	0.478	**0**
Piped drinking water	0.778	**0**
Delayed presentation (days)	- 0.272	**21**
Temperature (°C)	0.418	**38.0**
Dysgeusia	- 1.726	**1**
Headache	1.119	**0**
Arthralgia	1.803	**0**
Low CRP	0.994	**0**
Constant	- 20.489	
Score	**- 11.771**	
Probability	**0.000**	

Table 3.3.2.10. Scoring algorithm for a confirmed chikungunya case (R269)

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Age (years)	0.017	**34**
Male	0.478	**0**
Piped drinking water	0.778	**1**
Delayed presentation (days)	- 0.272	**2**
Temperature (°C)	0.418	**38.9**
Dysgeusia	- 1.726	**0**
Headache	1.119	**1**
Arthralgia	1.803	**1**
Low CRP	0.994	**1**
Constant	- 20.489	
Score	**0.4992**	
Probability	**0.622**	

Table 3.3.2.11. Scoring algorithm for a confirmed non-chikungunya case (R006)

Predictor	Weighting (LR Coefficient = B)	Score (0 = no, 1 = yes)
Age (years)	0.017	**37**
Male	0.478	**0**
Piped drinking water	0.778	**0**
Delayed presentation (days)	- 0.272	**5**
Temperature (°C)	0.418	**38.3**
Dysgeusia	- 1.726	**1**
Headache	1.119	**1**
Arthralgia	1.803	**1**
Low CRP	0.994	**1**
Constant	- 20.489	
Score	**- 3.0206**	
Probability	**0.047**	

Although the maximum and minimum probabilities generated appear impressive, a cut-off for the probability needs to be identified that will give acceptable levels of both sensitivity and specificity for a clinical prediction rule. This is unlikely to be the intuitive level of 0.5 and so an appropriate cut-off was sought using ROC curve analysis. Using the LR coefficients produced (labelled as "B" values in the tables shown), probabilities were calculated and used to construct ROC curves for the original data and each of the 5 imputations. All ROC curves for the imputed datasets were similar and so one was chosen at random (Imputation 1) for further analysis. The ROC curves for the original data and this imputed data set are shown in Figures 3.3.2.1 and 3.3.2.2.

Figure 3.3.2.1. ROC curve for diagnosing chikungunya from nine selected variables using the original data set

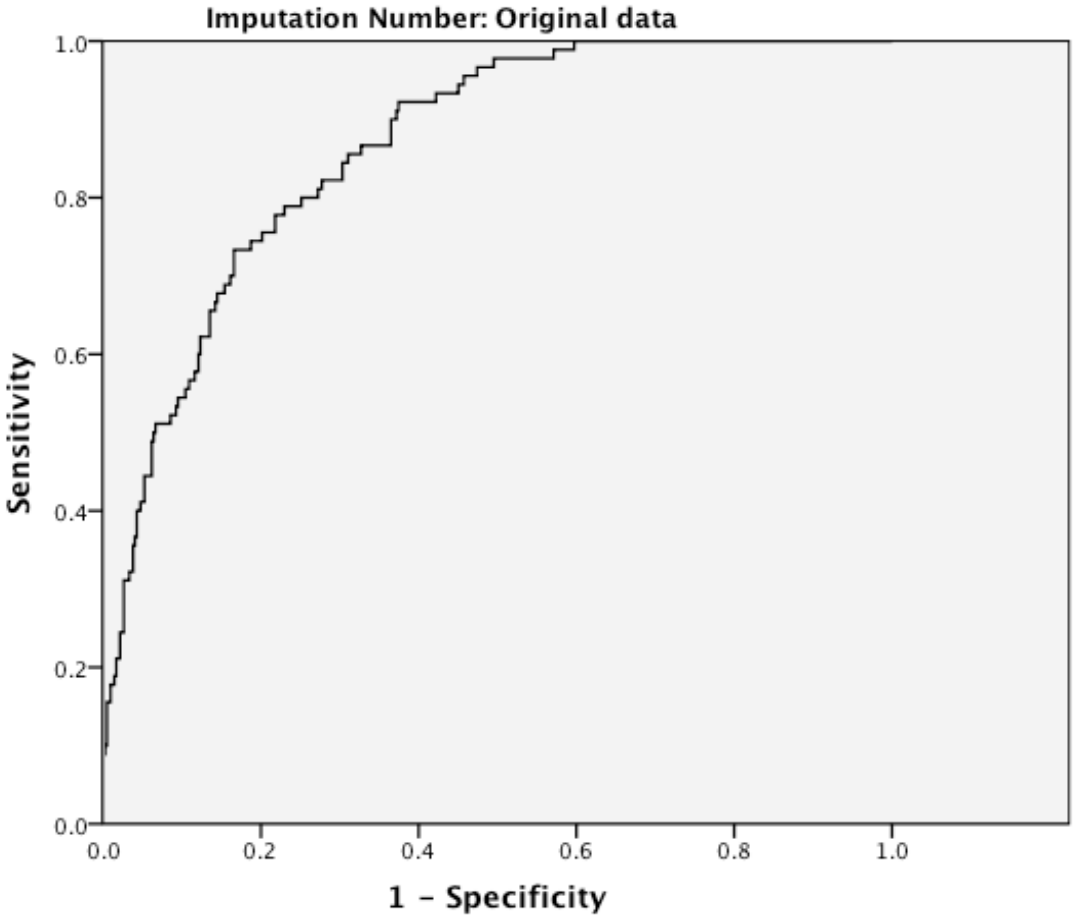
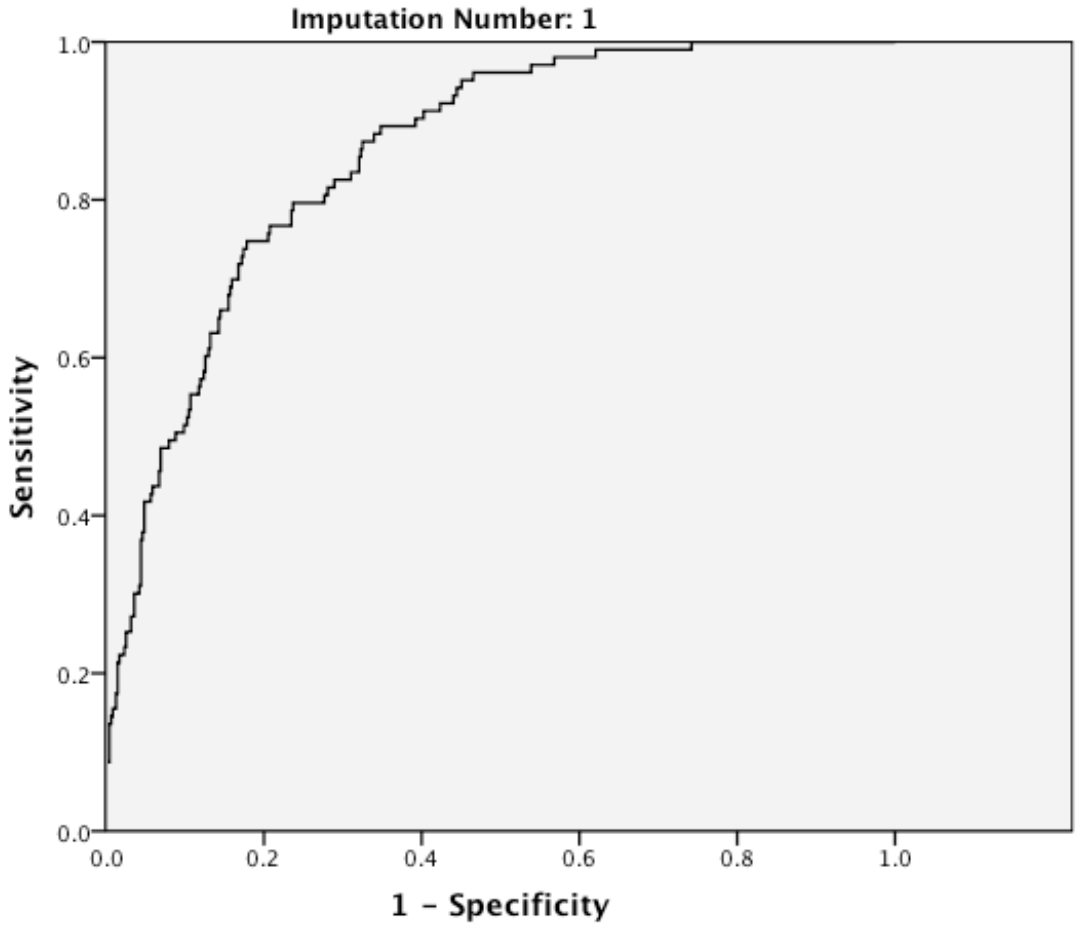


Figure 3.3.2.2. ROC curve for diagnosing chikungunya from nine selected variables using an imputed data set



Examination of the pivot table co-ordinates for these ROC curves shows that the following cut-offs will produce the following sensitivities and specificities :

Original Data :

Cut-Off = 0.0698457 : Sensitivity = 0.956 : Specificity = 1 – 0.457 = 0.543
Cut-Off = 0.5058091 : Sensitivity = 0.411 : Specificity = 1 – 0.047 = 0.953

Imputation 1 Data :

Cut-Off = 0.0808780 : Sensitivity = 0.951 : Specificity = 1 – 0.451 = 0.549
Cut-Off = 0.0987251 : Sensitivity = 0.903 : Specificity = 1 – 0.392 = 0.608

Cut-Off = 0.1557211 : Sensitivity = 0.806 : Specificity = 1 – 0.277 = 0.723
Cut-Off = 0.2476693 : Sensitivity = 0.709 : Specificity = 1 – 0.168 = 0.832
Cut-Off = 0.3197581 : Sensitivity = 0.602 : Specificity = 1 – 0.126 = 0.874
Cut-Off = 0.4210010 : Sensitivity = 0.505 : Specificity = 1 – 0.088 = 0.912
Cut-Off = 0.4929564 : Sensitivity = 0.417 : Specificity = 1 – 0.050 = 0.950

None of these probability cut-offs have both the sensitivity and specificity required for use in diagnosing chikungunya. However, the sensitivity and specificity values shown above indicate that the probability score for an individual patient based on this scoring system could offer some diagnostic information if taken in conjunction with other information available for that patient (but is not sufficiently accurate to form the basis of a diagnosis on its own).

3.4 Evaluation of Commercial Diagnostics Tests for Use in Sri Lanka

Dengue and chikungunya were each responsible for >50 confirmed cases and so attempts were made to evaluate commercial diagnostic tests for these infections. This was done prospectively for dengue using a single rapid diagnostic serology test (Section 3.4.1) and then retrospectively with a larger number of serology and antigen detection tests (Section 3.4.2). Evaluation of a commercial diagnostic test for chikungunya was also done retrospectively (Section 3.4.3) because a suitably large number of cases had not been expected and there were no commercial diagnostic tests available until 2010.

3.4.1 Prospective Use of a Commercial Diagnostic Test for Dengue

The Panbio Dengue Duo Cassette proved simple to use, but equivocal results (where the bands were very faintly visible in bright light) were common despite repeat testing of samples when such results occurred (Figure 3.4.1). Using the IgM band as an indicator of acute infection produced the results shown for admission samples in Table 3.4.1.1 and follow-up samples in Table 3.4.1.2. The diagnostic indices are shown in Table 3.4.1.3 and these improved overall (especially the sensitivity) when equivocal results were considered as positives.

Figure 3.4.1. Positive (+), weak/equivocal (w) and negative (-) bands on the Panbio Dengue Duo Cassette serology test used in this study

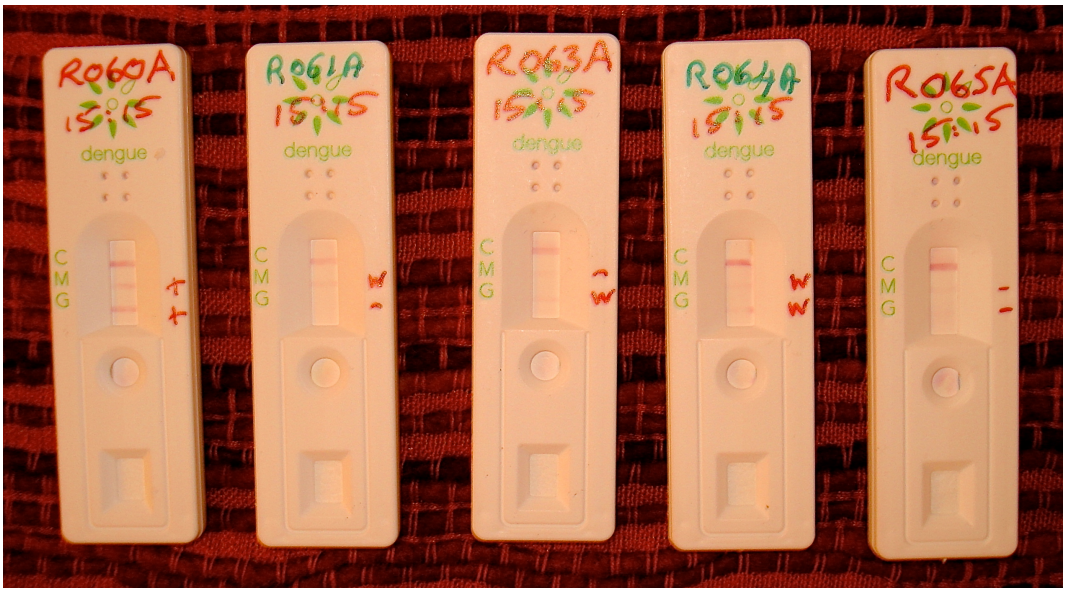


Table 3.4.1.1. Results of the Panbio Dengue Duo Cassette on admission

	Dengue	**Not Dengue**	**Total**
Test Positive	39	29	68
Test Equivocal	20	20	40
Test Negative	77	390	467
Test Not Done	1	41	42
Total	137	480	617

Table 3.4.1.2. Results of the Panbio Dengue Duo Cassette on follow-up

	Dengue	**Not Dengue**	**Total**
Test Positive	26	13	39
Test Equivocal	11	8	19
Test Negative	37	203	240
Test Not Done	63	256	319
Total	137	480	617

Table 3.4.1.3. Diagnostic performance of the Panbio Dengue Duo Cassette

	POS v NEG (Admission)	POS v NEG (Follow-Up)	POS+EQ v NEG (Admission)	POS+EQ v NEG (Follow-Up)
Sensitivity (95% CI)	33.6% (27.0–39.9%)	41.3% (31.9%–49.1%)	43.4% (36.7%–49.8%)	50.0% (40.8%–58.1%)
Specificity (95% CI)	93.1% (91.2–94.8%)	94.0% (91.2–96.3%)	88.8% (86.8–90.8%)	90.6% (87.6–93.3%)
PPV (95% CI)	57.4% (46.0–68.0%)	66.7% (51.5–79.4%)	54.6% (46.2–62.7%)	63.8% (52.1–74.1%)
NPV (95% CI)	83.5% (81.9–85.1%)	84.6% (82.1–86.7%)	83.5% (81.6–85.4%)	84.6% (81.8–87.1%)
LR+ (95% CI)	4.858 (3.076–7.678)	6.857 (3.641–13.211)	3.887 (2.769–5.429)	5.333 (3.290–8.678)
LR– (95% CI)	0.713 (0.634–0.801)	0.625 (0.528–0.747)	0.637 (0.553–0.730)	0.552 (0.449–0.676)

Combining positive and equivocal results for the admission results correctly classified seven (77.8%) of nine primary infections and 28 (66.7%) of 42 secondary infections. Using the same approach for follow-up results led to correct classifications in three (30.0%) of 10 primary infections and 24 (92.3%) of 26 secondary infections.

In confirmed dengue cases a down-grading of the IgM result on the Panbio Dengue Duo Cassette was noted between admission and follow-up samples. From 24 cases who were IgM positive on admission and also provided follow-up samples, there were 10 (41.7%) who became IgM negative and four (16.7%) who became IgM equivocal. Similarly, from 13 cases who were IgM equivocal on admission, there were eight (61.5%) who became IgM negative.

3.4.2 Retrospective Use of Commercial Diagnostic Tests for Dengue

From the 259 cases used in this evaluation, there were 99 (38.2%) with a confirmed diagnosis of acute dengue infection based on reference serology tests. The diagnostic performance of the commercial assays compared to the reference AFRIMS serology assays are shown in Table 3.4.2.

Table 3.4.2. Diagnostic performance of commercial dengue assays

Assay Type	Assay Brand	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
IgM Antibody Detection	Biosynex	79.8% (71-87%)	46.3% (38-54%)	49.9% (40-56%)	78.7% (69-87%)
	Merlin	72.7% (63-81%)	73.8% (66-80%)	63.2% (54-72%)	81.4% (74-87%)
	Panbio	70.7% (61-79%)	80.0% (73-86%)	68.6% (59-78%)	81.5% (75-87%)
	Standard Diagnostics	79.2% (71-87%)	89.4% (84-94%)	82.3% (73-89%)	87.7% (82-92%)
NS1 Antigen Detection	Bio-Rad	58.6% (48-68%)	98.8% (96-100%)	96.7% (86-100%)	79.4% (73-85%)
	Panbio	58.6% (48-68%)	92.5% (87-96%)	82.9 (72-91%)	78.3% (72-84%)
	Standard Diagnostics	48.5% (39-59%)	99.4% (97-100%)	98.0% (89-100%)	75.7% (69-81%)
Combined IgM & NS1 Detection	Panbio	89.9% (82-95%)	75.0% (68-82%)	69.0% (60-77%)	92.3% (86-96%)
	Standard Diagnostics	92.9% (84-97%)	88.8% (83-93%)	83.6% (75-90%)	95.4% (91-98%)

Further analysis showed that IgM detection in dengue cases increased from 0% on the first day of illness to 90% on the 10th day, whereas NS1 detection decreased from 100% on the first day of illness to 20% on the 10th day (Blacksell *et al.*, 2011b). This explains why the diagnostic performance of the combined IgM & NS1 detection tests was superior to that of each separate test.

3.4.3 Retrospective Use of a Commercial Diagnostic Test for Chikungunya

From the 292 paired samples used in this evaluation, there were 52 (17.8%) with a confirmed diagnosis of acute chikungunya infection based on reference serology & RT-PCR tests. The diagnostic performance of the commercial Standard Diagnostics (SD) assays and the reference AFRIMS serology and RT-PCR assays are shown in Table 3.4.3.

Table 3.4.3. Diagnostic performance of commercial chikungunya assays

Assay	Sample (n=292)	Sensitivity (Cases/Total) (95% CI)	Specificity (Cases/Total) (95% CI)	PPV (Cases/Total) (95% CI)	NPV (Cases/Total) (95% CI)
SD IgM ICT	Acute	3.9% (2/52) (0-13%)	95.0% (228/240) (91-97%)	14.3% (2/14) (2-43%)	82.0% (228/278) (77-86%)
SD IgM ELISA	Acute	3.9% (2/52) (0-13%)	92.5% (222/240) (88-96%)	10.0% (2/10) (1-32%)	81.6% (222/272) (77-86%)
SD IgM ELISA	Convalescent	84.1% (44/52) (72-93%)	91.3% (219/240) (87-95%)	67/7 (44/65) (55-79%)	96.5 (219/227) (93-99%)
AFRIMS HAI	Acute	21.2% (11/52) (11-35%)	95.0% (228/240) (92-98%)	47.8% (11/23) (27-69%)	84.8% (228/269) (80-89%)
AFRIMS IgM EIA	Acute	21.2% (11/52) (11-35%)	95.4% (229/240) (92-98%)	50.0% (11/22) (28-72%)	84.8% (229/270) (80-89%)
AFRIMS RT-PCR	Acute	88.5% (46/52) (77-96%)	100% (0/240) (99-100%)	100% (46/46) (92-100%)	97.5% (238/244) (95-99%)

Further analysis confirmed that IgM detection in chikungunya cases by the commercial tests was very poor within the first week of illness even when compared to the AFRIMS serology tests (Blacksell *et al.* 2011a). The high sensitivity of the AFRIMS RT-PCR for acute samples suggests that more direct diagnostic methods (such as antigen detection) could also be useful in diagnosing acute chikungunya infection.

Chapter 4
Discussion

4. Discussion

This study was partly successful in achieving its three aims (Section 1.1.1) :

1. The causes of febrile illness were identified in 62.8% of a representative sample of patients admitted to hospital over a 1-year period and this was despite the large number of different diseases identified, a follow-up rate of 48.6% that compromised paired serology tests and non-optimised tests for leptospirosis and rickettsial infections.

2. Clinical prediction rules were developed that could diagnose the most common infections found (dengue and chikungunya), but these had low sensitivity (or low specificity if the probability cut-off was adjusted to improve sensitivity).

3. A rapid (point-of-care) diagnostic test for dengue fever was evaluated and found to have low sensitivity in acute samples, but this improved when the test was later repeated in conjunction with a new dengue antigen detection test. A chikungunya diagnostic test was also evaluated later and a collection of serum samples with confirmed diagnoses and extensive clinical data is available for future use.

4.1 Patient Recruitment, Data Collection & Sample Collection

Considerable efforts were made to ensure that this study was as representative as possible and patient recruitment ran smoothly over the 1-year period. The only exception to this was a 2-week period in April when monthly recruitment was reduced by ~28 cases (47.5%) compared to adjacent months (Table 3.1.1). Overall 86.8% of all eligible patients were recruited with no sex or age bias, from both urban and rural areas and the distribution of cases within Gampaha District (Figure 3.1.2.2) was similar to that of the population as a whole (Figure 3.1.2.3). However, febrile patients were significantly younger and more likely to be male (Section 3.1.1) and this sex bias has been reported in most previous fever studies from Asia [Murdoch *et al.*, 2004; Wagenaar *et al.*, 2004; Suttinont *et al.*, 2006; Joshi *et al.*, 2008; Chrispal *et al.* 2010]. It may be that younger males have less immunity and greater exposure to the most common infections (dengue, chikungunya and leptospirosis) found in this

part of Sri Lanka. However, it is also possible that older females are less likely to present to hospital with febrile illness and a community-based study would be necessary to assess this.

Although only 6.4% of all medical admissions had febrile illnesses, this number varied from 31 to 117 on a monthly basis, which accounts for most of the monthly variation in medical admissions. Not surprisingly, there were more admissions during and immediately after the rainy seasons (Figure 3.1.1.2) probably due to an increased risk of dengue, chikungunya and leptospirosis transmission. At such times the hospital resources became dangerously over-stretched and improving the effectiveness and efficiency of the management of febrile illnesses would help matters considerably.

Data collection proceeded smoothly and the Ragama Fever Study database of demographic, clinical, laboratory and diagnostic data may prove to be the greatest legacy of this study. Complex searches are easily performed and the results can then be exported to various statistical software packages for analysis. With 403 fields and 617 patient records there is great potential for further exploration of various epidemiological, clinical and diagnostic issues. The discovery that 48.0% of cases would have to be excluded from multiple logistic regression analyses (Sections 3.3.1 & 3.3.2) due to missing values was a great surprise considering how complete the data collection and entry appeared to be. This is partly explained by the large number of variables involved and the inclusion of laboratory results that were not performed on all patients as part of the study. (For example, it was expected that all febrile patients would have a full blood count, urea & electrolytes and liver function tests performed as part of their routine care, but this was not the case.) Using a smaller number of variables focussed on a smaller number of diseases would help to avoid missing values, but this study was always intended to be wide-ranging so that future studies could be more focussed.

Sample collection, processing and transportation also proceeded smoothly despite numerous logistical challenges. Admission blood samples were collected in 88.3–98.2% of cases, but collecting admission urine samples was only possible in 76.5% of cases since this required greater patient compliance (Table 3.1.3). Despite

maximal efforts the follow-up rate for patients was only 48.6% and ultimately paired samples for serology were available in 47.8% of cases. These figures are similar or superior to those described in previous studies of this type in Asia, for example 32-51% in Vietnam [Wagenaar *et al.*, 2004] and 13% in Nepal [Murdoch *et al.*, 2004]. Having such a low rate of follow-up and paired serum samples could lead to bias against diagnoses that resolve rapidly and completely and which require paired serology tests for diagnosis. Four sets of identical samples were produced for distribution to various international reference laboratories and one set was reserved for future studies in Sri Lanka.

4.2 Diagnoses

This study was successful in showing the wide range of infectious diseases that present with febrile illnesses in western Sri Lanka. Most cases (62.8%) had confirmed diagnoses, which is a reasonable result since the most common infections (dengue, chikungunya and leptospirosis) were mostly diagnosed from paired serology samples, which were only available in 47.8% of patients recruited. This confirmation rate is similar or superior to those described in previous studies of this type in Asia, for example 68% in Thailand [Suttinont *et al.*, 2006], 48% on the Thai-Burmese border [Ellis *et al.*, 2006] and 37% in Nepal [Murdoch *et al.*, 2004].

In retrospect it would have been useful to test for influenza and other viral and atypical respiratory infections because there was a marked increase in undiagnosed febrile illnesses (mostly acute respiratory infections) for a month following the Sinhalese / Tamil New Year on 14 April 2007. At this time of year people travel widely in Sri Lanka to be with their extended families and April to July is recognised as the time when most cases of influenza and other acute respiratory infections occur [Perera *et al.*, 2010], which also corresponds to one of the rainy seasons in the south-western “wet” zone of Sri Lanka (Figure 3.1.1.3).

Other viruses were not universally tested for because they were not expected to occur in adults presenting to hospital (*eg.* measles, mumps, rubella, EBV, CMV and HAV) or because their prevalence was known to be low in Sri Lanka at the time of the study (*eg.* HBV, HCV and HIV infection) as described in Section 1.2.14. Only a

single patient with previously-diagnosed HIV was recruited and subsequently diagnosed as having pancreatitis with no infectious cause identified in the study. One other patient with known HIV was admitted to the medical wards used during the study period, but was not febrile. Both patients had previously worked and been sexually active in the Middle East. Two of the study patients were tested for HIV infection by the local consultant physicians as part of their routine care and both were found to be negative.

Universal testing for filariasis was not included in this study for several reasons. Acute filariasis usually presents with obvious lymphatic or genital involvement rather than just an undifferentiated febrile illness. In Sri Lanka, brugian filariasis was previously found in certain parts of the North-Western, Eastern and Southern Provinces, whereas bancroftian filariasis was found in a narrow strip along the south-western coastline [Sasa, 1976]. However, vector control and mass drug administration with diethylcarbamazine (DEC) have now led to complete elimination of *Brugia malayi* [Gautamadasa, 1986] and a marked reduction in the prevalence of *Wuchereria bancrofti* [Wijegunawardana, 2012]. Universal laboratory testing of patients in this study with microfilarial blood films or DEC provocation tests would have been laborious in such a large number of patients and serology tests are not able to distinguish between acute and previous infections. There is a filarial antigen test available in an immuno-chromatic test (ICT) format, but patients are known to remain antigen positive for years after apparently successful treatment [Schuetz *et al.*, 2000] and so a positive result is not necessarily indicative of an acute infection. Therefore filariasis was not routinely tested for and no suspected cases were admitted during the study period.

No confirmed, indigenous malaria cases were identified in this study and there were also no confirmed (or suspected) cases of brucellosis or JEV infection. As described earlier, these have been significant causes of undifferentiated febrile illness in Sri Lanka in the past. However, they do not appear to have been a significant problem in Gampaha District during this study and so will not be discussed further.

4.2.1 Bacteraemias

Bacteraemia was found in only 4.2% of patients despite using two blood culture systems in parallel. There was no significant difference in culture positivity between the in-house bottles (IHBs) and BacT/ALERT bottles (BABs) despite the presence of antibiotic inhibitors in the latter. However, the contamination rate was significantly higher in the IHBs because the re-usable rubbers caps became damaged by repeated use. This has now led to a compromise solution in which non-reusable commercial bottles from a local supplier are used in the Department of Microbiology. These should produce similar positive culture rates, with fewer contaminants and at minimal additional cost compared to the previous IHB system.

There was excellent correlation between the work done at the University of Kelaniya and the University of Liverpool in identifying bacterial isolates and antibiotic sensitivities. The *B. pseudomallei* isolate was not identified locally due to known limitations with the microbial identification panels used [Inglis *et al.*, 2005; Brent *et al.*, 2007]. Cases of melioidosis continue to occur in Sri Lanka, but these are mostly reported in travellers who have the diagnosis made when they return to their countries of origin [Peetermans *et al.*, 1999; Jayasekara *et al.*, 2006; Hesstvedt *et al.*, 2011]. Although, there is a risk that cases of melioidosis will be undiagnosed or misidentified in Sri Lanka, other prospective studies have also found very few cases [Inglis *et al.*, 2008].

Acquired antibiotic resistance was found in 23.1% of bacterial isolates and these included quinolone resistance in *S. typhi* (quinolone resistance), co-amoxiclav and ESBL resistance in *E. coli* and ESBL resistance in *K. pneumoniae*. These resistance profiles could easily spread in Sri Lanka due to a lack of microbiology services, over-use of empirical antibiotics in hospitals and misuse of antibiotics in the community. For example, azithromycin was being heavily marketed for a wide range of infections (including upper respiratory tract infections) during the study period and was easily purchased over-the-counter from local pharmacies. The problem of antimicrobial resistance in Asia has reached prominence recently with the spread of New Delhi metallo-β-lactamase-1 (NDM-1) carbapenemase [Khan & Nordmann, 2012] and ESBLs such CTX-M-15 [Wickramasinghe *et al.*, 2012] in *E. coli* and *K.*

pneumoniae. Awareness of the problem and the means of preventing it seem to be increasing in Asia [WHO, 2011], but the role of microbiology services in guiding the use of antibiotics and infection control is worthy of further emphasis.

4.2.2 Tuberculosis

TB was probably under-represented in this study due to the proximity of the (National) Chest Hospital at Welisara. However, in this 1-year study there were 17 cases admitted to a quarter of the medical wards suggesting that there would be ~68 cases per year admitted to the Colombo North Teaching Hospital as a whole. Most of the cases seen had acute presentations, which may explain why TB was not suspected initially. Although TB is not considered to be a major problem in Sri Lanka [WHO, 2008], it still represents a major socio-economic burden for individuals and their families in this country [Morton-Bailey, 2010].

4.2.3 Leptospirosis

Although there were only 32 leptospirosis cases confirmed on gold standard investigations (culture or MAT serology), there were a further nine with equivocal serology results and another eight who were diagnosed clinically but not adequately investigated. Considering these diagnostic and sampling problems and also the fact that at least 43.0% of patients took antibiotics before admission, the true number of leptospirosis cases was probably significantly higher. These issues greatly impede the systematic study of leptospirosis in Sri Lanka, but could be partly overcome by using local *Leptospira* isolates in future MAT serology tests and an appropriate PCR test on admission.

MAT serology is known to have low sensitivity even with paired serum samples and is also unreliable at predicting the infecting *Leptospira* serovar in cases where an isolate is also obtained for further identification by CAAT [Smythe *et al.*, 2009]. This is often thought to be due to differences between the *Leptospira* serovars used in the MAT and those causing disease in a particular area. Therefore the use of region-specific or even patient-specific isolates have been recommended, but sometimes this seems to make little difference [Murray *et al.*, 2011]. There are now

several established PCR tests for diagnosing leptospirosis [Agampodi *et al.*, 2012] and although one might think that PCR testing would be difficult in developing countries, it can be easier to provide than leptospire culture and MAT serology facilities.

The identification of *Leptospira* isolates in this study was done using CAAT and MLST because the panel of serovars used in the MAT serology (performed in the UK) was not optimised for use in diagnosing leptospirosis from Sri Lanka. Although 13 (76.5%) of the 17 isolates were *L. interrogans* serovar Pyrogenes (Clonal Complex 12 on MLST), there were composed of four different sequence types (ST 49, 74, 75, 76) suggesting a stable endemic situation rather than an epidemic situation due to a single dominant clone. The remaining four isolates included two *L.* interrogans and two *L. borgpetersenii*, which were all undesignated with regard to serovar and two of these seem to be unique to Sri Lanka according to the MLST database (http://leptospira.mlst.net).

From the 32 confirmed cases it was possible to describe clinical and laboratory features (Table 3.2.6.3) for comparison with those reported previously in Sri Lanka (Tables 1.2.7.1 & 1.2.7.2). Earlier studies in Sri Lanka specifically recruited suspected leptospirosis cases based on the most characteristic features of the disease (*eg.* icterus and conjunctival suffusion) and so it is not surprising that these features were more common in their case series [Rajasuriya *et al.*, 1964; Ramachandran *et al.*, 1974]. The clinical and laboratory features found in my study (Table 3.2.6.3) suggest that the most characteristic features are less common if all febrile patients are recruited as this will reveal the full spectrum of disease caused by leptospirosis. Mild cases (possibly modified by pre-hospital antibiotic use) were often not suspected to be leptospirosis during this study despite the patient having significant risk factors for the disease. At the other end of the disease spectrum, leptospirosis was responsible for all ITU admissions, 35.7% of deaths with a mean age of 37.6 years compared to 73.8 years for deaths from other causes.

Even with only 32 confirmed leptospirosis cases (and possibly 17 unidentified cases in the control group) it was possible to prove a significant unadjusted association between leptospirosis and proteinuria on admission for patients in this study (Section

3.2.15). However, further statistical analysis will first require improved diagnostics to correctly identify true leptospirosis cases as described above. Although there is an emphasis on using gold standard laboratory diagnostics in studies such as this, there is also increasing recognition that using reference tests with such high specificities and low sensitivities represents a sort of "fool's gold" [Limmathurotsakul *et al.*, 2012]. Mathematical modelling using Bayesian latent class models enables the accuracy of diagnostic tests to be evaluated without the assumption that a gold standard reference test is perfect [Pan-Ngum *et al.*, 2012]. The leptospirosis data (especially the IgM ELISA screening test and equivocal MAT serology results) in this study may well benefit from being re-assessed in this way.

Identifying more leptospirosis cases using non-gold standard tests and mathematical modelling would enable more samples from this study to be used for evaluating diagnostic tests in the future. The latest commercial serology test for leptospirosis (the Panbio *Leptospira* IgM ELISA) is reported to have only 76.1% sensitivity and 82.6% specificity [Desakorn *et al.*, 2012] and this would need to be re-evaluated using local samples before it could be recommended for use in Sri Lanka.

4.2.4 Q Fever

Acute Q fever was diagnosed in 17 (2.8%) patients using serology tests at the highly-respected WHO reference laboratory in Marseilles, France. The definition of acute Q fever included patients who had anti-phase II antibody titres that were ≥200 for IgG and ≥50 for IgM at any time. My subsequent experience of managing >60 acute Q fever cases from the point of seroconversion onwards [Bailey *et al.*, 2011 & unpublished data] shows that nearly all acute cases are sero-negative on admission (but then seroconvert after ~2 weeks) and the titres used in the Marseilles definition are often found for up to six months after acute Q fever infection. Therefore this definition for acute Q fever is probably over-sensitive for clinical studies such as mine and this is also suggested by the findings that 50.0% of Q fever cases identified had co-infections and only 23 previous Q fever cases were identified compared to 18 active cases (despite the fact that previous infections are usually identifiable years later). A recent publication using the same serology and definitions reported acute Q fever in three (1.6%) patients suspected of having rickettsial infections in southern

Sri Lanka, but paired serology results were only available in one case [Angelakis *et al.*, 2012]. Using additional tests (*eg.* Q fever PCR) or alternative Q fever serology assays (*eg.* validated commercial ones) would help to clarify this matter and these tests could be applied retrospectively to the Ragama Fever Study samples.

4.2.5 Rickettsial Infections

Rickettsial infections were diagnosed in 14 (2.3%) of patients and this was also based on serology tests only, which are an indirect method of diagnosis and can be negative at presentation and positive for weeks following an acute infection. Once again there was a high rate of co-infections (35.7%) and a large proportion of "probable" (possibly previous) infections (42.9%). Although the diagnosis of a *R. conori* subspecies *indica* infection was robust, the diagnosis of other rickettsial infections was uncertain.

This was most obvious for scrub typhus because eschars are usually present and do not occur in the typhus fevers or spotted fever due to *R. conorii* sub-species *indica* [La Scola *et al.*, 2009]. The Marseille scrub typhus serology showed low sensitivity and specificity for suspected cases with eschars and a previous study from the same laboratory did not find any cases of scrub typhus in central Sri Lanka [Nagalingam *et al.*, 2009] even though scrub typhus had been reported there previously [Kularatne *et al.*, 2003]. The Bangkok scrub typhus serology results correlated better with the presence or absence of eschars and combining the positive results from each laboratory would have identified all cases that presented with eschars. These discrepancies should be discussed between the two laboratories and it may be that there was variation in the antigens used and that these require optimisation for use in Sri Lanka. A recent study in southern Sri Lanka using the Marseille scrub typhus serology reported that this was the most common rickettsial infection found [Angelakis *et al.*, 2012] and so it may be that the problem has been resolved already. In future more direct methods of diagnosis (*eg.* biopsy and PCR of eschars) should be used in parallel with serology to help clarify the diagnostic accuracy of reference and commercial serology tests for scrub typhus [Koh *et al.*, 2010].

The exact nature of the *Rickettsia* species causing spotted fever in Sri Lanka has been elusive because the necessary range of antigens to determine this have rarely been used. Only one other study from Sri Lanka has used a full range of spotted fever antigens (also from Marseille) and no cross-adsorption western blotting results were reported [Angelakis *et al.*, 2012]. Other recent studies have identified spotted fever cases using undefined spotted fever antigens [Liyanapathirana & Thevanesam, 2011], *R. rickettsi* (Rocky Mountain spotted fever) antigen [Reller *et al.*, 2012b] or generic *R. conorii* antigen [Premaratna *et al.*, 2010] only. The identification of a *R. conorii* subsp. *indica* in this study is a significant step forwards, but direct identification of the *Rickettsia* species responsible for spotted fever in Sri Lanka (by culture or PCR) is still necessary to optimise diagnostic tests for this rickettsial infection in this country.

As well as using non-optimised diagnostic tests, other studies of rickettsial infection in Sri Lanka have usually recruited selected patients only using various clinical definitions (*eg.* those with a rash and/or eschar and/or prolonged fever and/or prompt response to doxycycline or chloramphenicol), which makes it difficult to assess the true frequency of these infections. However, a recently published study from southern Sri Lanka did perform rickettsial serology tests on a large group of unselected febrile patients over an eight-month period [Reller *et al.*, 2012b]. This found that 17.7% of cases had acute rickettsioses of whom ~69% had a spotted fever, ~23% had a typhus fever and ~7% had scrub typhus, but these unexpected results are difficult to interpret since the tests used were not optimised for Sri Lanka. Overall, there has been a consensus developing in previous papers that spotted fever is most common in the central highland areas and scrub typhus is most common in more coastal lowland areas, but even this is not certain due to the relatively poor quality of the studies performed so far.

Rickettsial infections remain a significant cause of febrile illness in Sri Lanka and are very difficult to study due to a lack of optimised diagnostic tests. More detailed studies are required and in the meantime local clinicians should always consider this diagnosis in patients with undifferentiated febrile illnesses and consider empirical treatment with doxycycline, azithromycin or chloramphenicol.

4.2.6 Dengue

Dengue is endemic in Sri Lanka, as shown by official notifications of cases (Tables 1.2.10.1 & 3.1.2.5) and occurs all year round with peaks in the rainy seasons, as shown by the monthly frequencies of confirmed cases during this study (Figure 3.1.1.3). All four genotypes were identified with a predominance of DENV-3 and DENV-2, which has also been the pattern in previous years [Kanakaratne *et al.*, 2009] since the emergence of a new DENV-3 genotype III (Indian sub-continent) clade in 2003 [Messer *et al.*, 2003]. This clade is thought to have originated in East Africa, which is also known to be the source of the chikungunya epidemic that spread through the Indian Ocean area in 2006-8 [Staples *et al.*, 2009]. DHF occurred in only four (2.9%) of the confirmed dengue infections and two of these were RT-PCR with both identified as DENV-3.

The diagnosis of dengue was greatly helped by the availability of validated serology tests and an RT-PCR test, which was especially useful when only acute/admission serum samples were available. Without the RT-PCR tests there would have been only 101 dengue cases (73.7%) identified instead of 137 (Section 3.2.9).

There were 10 cases of dengue and chikungunya co-infection, which is not surprising since both are transmitted at the same time of year by the same *Aedes albopictus* mosquitoes and co-infections have been reported previously [Chang *et al.*, 2010].

Overall the clinical and laboratory features on admission for confirmed dengue cases from this study (Table 3.2.9.3) are similar to previous reports in Sri Lanka [Kularatne *et al.*, 2005; Malavige *et al.*, 2006]. Although my data adds more detail, it also confirms the non-specific presentation of dengue compared to other undifferentiated febrile illnesses (*eg.* chikungunya and leptospirosis) in this part of Sri Lanka. This is why the clinical and laboratory features were subjected to multiple logistical regression analysis and a clinical prediction rule was developed (Section 3.3.1), which is discussed later (Section 4.3). A rapid diagnostic test for dengue that was suitable for use in Sri Lanka would also overcome this problem and one was evaluated in this study (Section 3.4.1), which is also discussed later (Section 4.4).

4.2.7 Chikungunya

An outbreak of chikungunya began in Sri Lanka in 2006 with the most cases occurring during the rainy season in October – December 2006, as shown by the epidemic curve of confirmed cases during this study (Figure 3.1.1.4). Not surprisingly the CHIKV strain identified was identical to that found elsewhere in the Indian Ocean area from 2006-8 and very similar to that found there in 2005-7 and also similar to the Central Africa strains from which it is thought to have originated (Figure 3.2.11).

The diagnosis of chikungunya was also greatly helped by the availability of validated serology tests and an RT-PCR test, which was especially useful when only acute/admission serum samples were available. Without the RT-PCR tests there would have been only 52 chikungunya cases (50.5%) identified instead of 103 (Section 3.2.11). The apparently long viraemia of chikungunya makes it especially amenable to RT-PCR diagnosis.

Persistent arthralgia for >2 weeks was found in 27 (52.9%) of 51 chikungunya cases who attended for follow-up, but there is likely to be some positive selection bias in this since people with persistent arthralgia would be more likely to attend.

Overall the clinical and laboratory features on admission for confirmed chikungunya cases from this study (Table 3.2.11.2) are similar to previous reports from the Indian Ocean epidemic in 2004-8 [Staples *et al.*, 2009; Burt *et al.*, 2012]. Although my data adds some detail, it also confirms the non-specific presentation of chikungunya compared to other undifferentiated febrile illnesses (*eg.* dengue and leptospirosis) in this part of Sri Lanka. This is why the clinical and laboratory features were subjected to multiple logistical regression analysis and a clinical prediction rule was developed (Section 3.3.1), which is discussed later (Section 4.3). A rapid diagnostic test for chikungunya that was suitable for use in Sri Lanka would also overcome this problem and one was evaluated using the data and samples from this study at a later date, which is also discussed later (Section 4.4).

4.2.8 Hantavirus Infection

Although 25 (8.5%) of 295 patients with paired samples had evidence of previous hantavirus infection using an IgG screening ELISA, this number fell to 18 (6.1%) when a serology gold standard IgG/IgM IFA was used and only six (2.0%) of these were also IgM ELISA positive of whom only one (0.3%) showed a greater than four-fold increase in IFA titres to confirm an acute infection. Therefore hantavirus infection seems to be uncommon in this part of Sri Lanka, but the discovery by RT-PCR of a possibly novel hantavirus related to THAIV is the first direct evidence of human hantavirus infection in Sri Lanka and also the first direct evidence of human infection with a THAIV-like hantavirus. This is of considerable scientific and medical interest and should help in choosing and designing hantavirus diagnostic tests for future use in Sri Lanka.

The International Committee on the Taxonomy of Viruses (www.ictvonline.org) requires that new hantaviruses have a ≥7% difference in amino-acid identity compared with previously identified complete S and M segment gene sequences, a ≥4-fold difference in the results of 2-way cross-neutralization tests using viral cultures, a unique ecologic niche such as a different primary rodent/insectivore reservoir and no natural re-assortants with other species [Plyusnin *et al.*, 2011]. These are demanding criteria and not enough data is available for the hantavirus identified in this study to meet them at present. However, the partial M segment sequence data did show only 83% genetic homology (a 17% difference) with the nearest known hantavirus relative (THAIV) and similar results (admittedly with more sequence data) have been considered evidence of novel hantavirus genotypes in the past [Plyusnina A *et al.*, 2009; Cruz *et al.*, 2012]. Therefore I suggest that the hantavirus identified in this study is a novel genotype most closely related to THAIV and propose that it be called Ragama virus (RAGV).

A more recent study in the Central Province of Sri Lanka recruited 105 patients with suspected leptospirosis or hantavirus infection and found that 8 (7.6%) had positive hantavirus serology [Gamage *et al.*, 2011]. Overall these titres were highest against THAIV, then SEOV and then HTNV and so these findings are consistent with my own. Until now THAIV and related viruses, such as Serang virus (SERV) [Plyusnina

et al., 2009], have only been identified in rodents and there has been only indirect serological evidence and limited clinical evidence of human infection [Pattamadilok *et al.*, 2006; Chandy *et al.*, 2009b; Gamage *et al.*, 2011]. The patient (R504) from whom this THAIV-like hantavirus (RAGV) was identified had an illness that was compatible with a mild hantavirus infection and no other infectious disease was diagnosed from the extensive microbiological investigations used in this study. The patient's previous splenectomy could have contributed to his illness (although this would be unusual for a viral illness) and the fact that the virus was detectable 3 days after the onset of symptoms.

Since the reservoir hosts for THAIV and SERV are known to be the greater bandicoot rat (*Bandicota indica*) and the Asian house rat (*Rattus tanezumi*) respectively, it would be worth trying to isolate hantaviruses from similar rodent species in Sri Lanka to see how they compare with the hantavirus (RAGV) identified in this study. Bandicoot rats are known locally in Sinhalese as the "Uru-Meeya" or "Pig-Rat" and also include the lesser bandicoot rat (*Bandicota bengalensis*).

4.2.9 Co-Infections

Co-infections are a common finding in comprehensive studies of undifferentiated febrile illnesses in the tropics and sub-tropics and do raise questions regarding false positive results, which could arise due to cross-reactions in PCR or serology tests or else persistent antibody titres in serology tests. Co-infections were reported in 28 (3%) of 876 patients in Patan, Nepal [Murdoch *et al.*, 2004], 19 (18%) of 103 patients in Dhulikhel, Nepal [Blacksell *et al.*, 2007], 34 (6%) of 613 patients on the Thai-Burmese border [Ellis *et al.*, 2006] and 112 (13%) of 845 patients in Thailand [Suttinont *et al.*, 2006]. In this study co-infections were found in 30 (4.9%) of 617 patients, which is similar or less than in previous studies. The most common co-infection was dengue and chikungunya (involving 10 of 137 dengue and 103 chikungunya patients), which is epidemically very plausible and the diagnoses were made by a combination PCR and well-defined serology tests. The next most common co-infections were Q fever with various other infections (involving 9 of 18 Q fever cases) and rickettsial infections with various other infections (involving 5 of 14 rickettsial infection cases). These results are of greater concern because they are

unexpectedly frequent, epidemiologically less plausible and both Q fever and rickettsial infections were diagnosed by serology tests alone.

4.2.10 Quality Assurance for Reference Laboratories

One rarely questions the accuracy of diagnostic results from reference laboratories and this is understandable considering their expertise, internal validation systems, publications, prestige and generosity in supporting the work of others. Hence their results are rarely compared with those of other reference laboratories and this is especially true for the most rare infections found in this study. However, questions do arise because of the discrepancies in scrub typhus results between Marseille and Bangkok, especially regarding patients with eschars (Sections 3.2.8 & 4.2.5) and also due to the unexpectedly high number of co-infections involving Q fever and rickettsial infections (Section 4.2.9). These problems are partly due to the fact that these diagnoses were made using serology tests only, which can take more than two weeks to become positive and may then remain positive for many months. Also a recent comparison of Q fever serology results between three different international reference laboratories has shown significant differences [Healy *et al.*, 2011]. Possible solutions to these problems include greater co-operation between reference laboratories (*eg.* through a quality assurance system similar to the Clinical Pathology Accreditation scheme in the UK) and the development of more direct diagnostic methods (*eg.* PCR and antigen detection tests) for these particular infections.

4.3 Clinical Prediction Rules

Previous fever studies in south and south-east Asia have usually concentrated on microbiology findings and paid less attention to correlating the diagnoses made with clinical findings (Section 1.3). Even when logistic regression has been performed the results have usually been unhelpful [Murdoch *et al.*, 2004; Ellis *et al.*, 2006] and one is left wondering if enough attention has been give to this issue or if useful prediction rules are an impossibility with such a wide range of undifferentiated febrile illnesses. Developing such rules for the most common infections identified was one of the aims of my study and the final results were disappointing because the diagnostic performance of the logistic regression model and clinical prediction rules were poor

and deteriorated significantly as they were refined using imputed data and selected variables (Tables 3.3.1.7 & 3.3.2.7).

The clinical prediction rules for both dengue and chikungunya had poor sensitivities (with poor PPVs) and good specificities (with good NPVs) when using the default probability cut-off of 0.5. One instinctively looks for a high sensitivity (with a low false negative rate) in screening assessments such as these because a negative result will be very good at ruling out disease and a positive result will usually be further investigated with more specific laboratory tests. The utility of a high specificity (with a low false positive rate) is perhaps less obvious, but a positive result will be very good at ruling in disease. Therefore the high specificity of the prediction rules could be used to identify patients with a very high chance of either dengue or chikungunya for early discharge from hospital, assuming that there were no complications. Alternatively, the rules could be used to identify cases for further study (*eg.* surveillance of viral genotypes), assuming that cases identified by the rule were representative of all cases.

Obviously, both good sensitivity and specificity are desirable, but the ROC curve analysis shows that this could not be achieved and so one must choose either a high sensitivity (by moving the probability cut-off) or high specificity (by using the default probability cut-off). The clinical prediction rules developed here can be used with a high sensitivity cut-off, but the resulting poor specificity (with a high false positive rate) would make this of limited value in defining suspected cases for further diagnostic testing.

One reason for the poor results was the amount of missing data, which required imputation before analysis could be performed and this led to a loss of diagnostic accuracy (Tables 3.3.1.7 & 3.3.2.7). The missing data is mostly explained by the very large number of variables involved in the database (403 in total, of which 59 recorded at the point of admission were used in the logistic regression model) and also by the use of laboratory results that were expected from all patients as part of their routine clinical management, but which were often not done. In future a smaller number of variables could be used and these should be chosen for a specific disease by considering the variables that were significantly associated with it in this study.

This smaller amount of data should then all be collected as part of that study without relying on data obtained from the routine management of patients.

Another limitation of the clinical prediction rules developed in this study is that they are based on data obtained during the chikungunya outbreak in Sri Lanka from 2006-7 and the number of cases seen each year is thought to have decreased dramatically since then. This is important for statistical reasons because higher prevalence leads to increased sensitivity and PPV, but decreased specificity and NPV. With similar numbers of dengue and chikungunya patients included, the rules should be good at distinguishing between these infections, which have very similar clinical features at presentation. However, they are not optimised for use when dengue is much more common than chikungunya, which is the normal epidemiological situation in Sri Lanka. In view of their similar clinical features, one could speculate that the dengue clinical prediction rule might have performed better if less chikungunya cases had been seen. Seasonal variation can also create problems for clinical prediction rules, which is one reason why this study was conducted over a complete year. The rules developed were not analysed for any seasonal variation because Sri Lanka has two variable rainy seasons each year (Figure 3.1.1.5) and arboviral infections occur throughout the whole year (Figure 3.1.1.3). Therefore it would be difficult, imprecise and rather artificial to create rules that worked for specific seasons only. Overall, variations in epidemiology pose a greater problem for the use of diagnostic rules compared to laboratory diagnostics.

The fact that co-infections occurred in 18 (13.1%) of 137 dengue cases and 13 (12.6%) of 103 chikungunya cases raises the question as to whether these cases should have been excluded during the development of clinical prediction rules. Ultimately, it was decided to include these cases because the majority were thought be genuine (including 10 cases of dengue/chikungunya co-infection) and any rules that were successfully developed would need to be able to reflect this fact. Also the omission of these cases from the analysis would probably have further compromised the statistical significance of the rules developed.

4.4 Commercial Diagnostic Tests

The Panbio Dengue Duo Cassette performed poorly with both admission and follow-up samples in comparison to the AFRIMS serology and RT-PCR tests used to define true dengue cases in this study. Its diagnostic performance (especially sensitivity) improved from admission to follow-up samples, which was expected since the onset-to-admission time in dengue is usually short and so even IgM antibodies may not have risen to the detection threshold in admission samples. However, the observed down-grading of the IgM result for this test from admission to follow-up samples shows that cases would have been missed if only follow-up samples were tested. Therefore both admission and follow-up testing is necessary to avoid missing cases that would otherwise be identified. This is an important point to remember for future clinical and research work since there may be a temptation to use the test only on admission or follow-up when resources are limited. The ability of the test to distinguish primary from secondary dengue infections was generally good, but primary infections on follow-up samples were often wrongly classified because IgG results are more likely to become positive (and be interpreted as secondary infections) with increasing time after the onset of infection.

Further evaluation of this Panbio Dengue Duo Cassette along with other dengue rapid diagnostic tests was performed using only those patients from this study who had paired serology samples and used only the AFRIMS serology (but not the RT-PCR) results to define confirmed dengue infections [Blacksell *et al.*, 2011b). In this select group the diagnostic indices (with 95% CIs) improved with sensitivity 70.7% (60.7–79.4%), specificity 80.0% (73.0–85.9%), PPV 68.6% (58.7–77.5%) and NPV 81.5% (74.6–87.3%), which compared favourably to other commercial point-of-care serology tests. Further improvements in diagnostic accuracy were also achieved by combining serology results with antigen detection of the NS1 antigen as described in Section 3.4.2. Another recent study that evaluated commercial IgM and NS1 detection ELISAs on admission samples has also reported similar results for the tests from Panbio (sensitivity 87.9% and specificity 84.5%) and Standard Diagnostics (sensitivity 87.4% and specificity of 95.6%) [Blacksell *et al.*, 2012].

It is not surprising that the diagnostic performance of these commercial assays improved when used on a select group of patients who had paired samples that were all positive on reference serology tests. Although this is a fair way to assess the performance of commercial assays, it does ignore the real-life occurrence of cases that are diagnosed without paired samples or by RT-PCR tests only, which probably explains why the Panbio Dengue Duo Cassette performed less well when used prospectively for all patients in this study. The issue of gold standard diagnostic tests having low sensitivity is now being investigated using Bayesian latent class models [Limmathurotsakul *et al.*, 2012] and so the data obtained from this study have also been used recently to estimate the true accuracy of diagnostic tests for dengue infection using the same approach [Pan-Ngum *et al.*, 2013].

In comparison, commercial diagnostic tests for chikungunya are much less developed and likely to remain so whilst there is less demand for them due to the epidemic (rather than endemic) nature of this disease. The earliest versions evaluated in this study performed poorly on acute samples, but so too did the reference serology test and only the RT-PCR test achieved acceptable results [Blacksell *et al.*, 2011a]. Similar results for serology assays have been reported since [Kosasih *et al.*, 2012] and there seems to be little prospect of an antigen detection test being developed at present.

4.5 Limitations & Further Studies

The main limitations of this study were the fact that it coincided with an epidemic of chikungunya, the 48.6% follow-up rate that compromised paired serology tests, the use of non-optimised diagnostic tests for use in Sri Lanka and the missing data that compromised the development of clinical prediction rules.

There is no way that the chikungunya epidemic could have been avoided when conducting this study, but it does mean that the epidemiology results and clinical prediction rules may not be applicable in future years. However, the fact that similar numbers of confirmed dengue and chikungunya cases were recruited did provide an opportunity to develop clinical prediction rules and evaluate diagnostic tests that would distinguish between them.

Low follow-up rates are known to be a major problem when conducting fever studies in developing countries and are especially significant due to the use of serology tests that require paired samples. This problem was anticipated at the beginning of the study and so travel expenses were paid to all those who attended follow-up and a reminder card was sent those who did not. Despite reinforcing these measures throughout the study, the follow-up rate remained ~50% throughout and this was probably due to the considerable distances that many patients had to travel to reach hospital and also because most patients in Sri Lanka attach little importance to clinical follow-up if their symptoms have resolved. Another recent fever study in Sri Lanka visited people at home to obtain follow-up data and samples if they did not attend for follow-up and were able to obtain paired samples in 82.4% of cases [Reller *et al.*, 2011], which seems to be a good idea for any future studies. Fortunately, the two most common infections found (dengue and chikungunya) were both identifiable by PCR tests performed in this study, but this is a circular argument since the availability of such tests ensured that these cases were identified.

There was no way to avoid the use of non-optimised tests for use in Sri Lanka during this study and this was a particular problem for leptospirosis and rickettsial infections. Hopefully, more appropriate tests can now be identified and developed using the data and samples obtained in this study. For leptospirosis the use of PCR tests and Bayesian latent class models to evaluate existing tests (*eg.* the screening IgM ELISA used) would be appropriate. For rickettsial infections there needs to be some harmonisation of the scrub typhus results obtained in the Marseille and Bangkok laboratories and tests for *Rickettsia conorii* subspecies *indica* should be developed and evaluated. More directs tests (*eg.* culture and PCR) need to be performed on suspected rickettsial infection patients in Sri Lanka to identify the exact species and strains involved so that diagnostic tests can then be improved.

Overall there was not a large amount of missing data for patients in this study, but there were a large number of patients with a small amount of missing data each, which interfered with the multiple logistic regression and clinical prediction rules. This occurred due to the large amount of data recorded on each patient and the use of "opportunistic results" that were generated by investigations used in the patients'

clinical management. In future it should be possible to study individual diseases by collecting a smaller amount of data on each patient and ensuring that all laboratory tests are performed as part of the study rather than as part of patients' expected clinical management.

Future studies in Sri Lanka on undifferentiated febrile illnesses would be well worth performing since there is such a wide range of causes and renewed interest in researching non-malarial acute undifferentiated fevers [Naing & Kassim, 2012]. Using the data and samples obtained in this study, it should be possible to identify selection criteria or commercial diagnostic tests that would allow suspected cases of individual diseases to be recruited into smaller and more focussed studies. Confirming these diagnoses would then need even greater efforts to obtain paired samples (*eg.* by performing home visits) and improved diagnostic tests that do not require paired samples (*eg.* PCR tests) would be especially useful. I believe that further studies on antibiotic resistance, tuberculosis, leptospirosis, Q fever, rickettsial infections and hantavirus infection would be the most worthwhile because these were the most difficult to diagnose in this study.

Chapter 5
References

5. References

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Chapter 6
Appendices

Appendix 1

Data Collection for a Fever Study in Sri Lanka

Last Updated 16 November 2006

See Appendix 2 for the consent form

Demographic details												
Name						BHT		UI [1]				
Address							Age		Sex			
GN division [2]						GN code [2]						
Number of years of education				Number of people in household								
Household income (LKR per month)				Patient is main income provider [3]						YES / NO		
Exposures within the previous 4 weeks												
Travel overseas	NO / YES =											
Close contacts affected [4]	household member / visitor to house / non-visiting neighbour / work contact / other											
	(give details + numbers)											
Place of work / occupation	animal farm / paddy field / other arable farm / handling fish / river / drains / sewers											
	other (give details)											
Water [5]	(please tick)	Boiled		Bottled		Tap		Well		Reservoir		Stream
	Drinking											
	Washing [6]											
	Bathing [7]											
Eating	uncooked vegetables [8]	never / monthly / weekly / daily										
	with unwashed hands [9]	never / monthly / weekly / daily										
Skin	usual footwear [10]	boots / closed shoes / open sandals / barefoot										
	any skin wounds [11]	YES / NO		Site								
Arthropods	visited scrub jungle [12]	YES / NO		Where								
	definite tick bite [13]	YES / NO		Site								
Animals [14]	(please tick)	None		Present in area		Present at house		Direct contact				
	Rodents											
	Dogs											
	Cats											
	Pigs											
	Cattle											
	Sheep											
	Goats											
	Horses											
	Birds											
Admission Data												
Name of hospital				Name of ward								
Date of arrival in hospital				Date of onset of illness [15]								
Other medical problems	(give details)											
Antibiotics before arrival	NO / unknown medicine / unknown antibiotic / YES =											
Previous typhoid vaccine	YES / NO / UNSURE			Previous JE vaccine				YES / NO / UNSURE				
Smoking consumption	_____ cigarettes / day			Alcohol consumption				_____ units / week				
Blood cultures sent	YES / NO			Labelling + date of cultures								
Heparinised blood sent	YES / NO			Labelling + date of blood								
Serum samples (admission)	YES / NO			Labelling + date of serum								
PCR sample sent	YES / NO			Labelling + date of sample								
Urine sample sent	YES / NO			Labelling + date of urine								

Appendix 1 **UI** _____

Clinical Features								
Fever (subjective) [16]	YES / NO / UNSURE			Fever (recorded) [17]	_____ °C or _____ °F			
Fever type [18]	continuous / intermittent / biphasic			Fever duration(s) [19]				
Other clinical features [20]	Y / N / U	Onset	Duration	Other clinical features [20]	Y / N / U	Onset	Duration	
Chills (cold sweats)	Y / N / U			Rigors	Y / N / U			
Wound	Y / N / U			Eschar	Y / N / U			
Skin flushing	Y / N / U			Skin rash	Y / N / U			
Icterus or Jaundice	Y / N / U			Haemorrhage or Bruising	Y / N / U			
Pruritis	Y / N / U			Lymphadenopathy	Y / N / U			
Conjunctival suffusion	Y / N / U			Conjunctival haemorrhage	Y / N / U			
Sore throat	Y / N / U			Abnormal taste	Y / N / U			
Headache	Y / N / U			Neck stiffness	Y / N / U			
Photophobia	Y / N / U			Focal neurological signs	Y / N / U			
Confusion	Y / N / U			↓ Glasgow Coma Scale [21]	/15			
Hypotension	Y / N / U			Myocarditis [22]	Y / N / U			
Chest pain	Y / N / U			Dyspnoea	Y / N / U			
Cough	Y / N / U			Sputum	Y / N / U			
Haemoptysis	Y / N / U			Weightloss (subjective)	Y / N / U			
Anorexia	Y / N / U			Constipation	Y / N / U			
Abdominal pain	Y / N / U			Abdominal distension	Y / N / U			
Nausea	Y / N / U			Vomiting	Y / N / U			
Diarrhoea	Y / N / U			Bloody diarrhoea	Y / N / U			
Hepatomegaly	Y / N / U			Splenomegaly	Y / N / U			
Oliguria < 400 ml/day	Y / N / U			Dysuria	Y / N / U			
Myalgia	Y / N / U			Arthralgia	Y / N / U			
Admitted to ITU	Y / N / U	Antibiotics given						
Other clinical features	(give details)							
Investigation results [23]	First	Worst	Date	Investigation results [23]	First	Worst	Date	
Haematocrit				Haemoglobin				
White cell count				Neutrophils				
Lymphocytes				Platelets				
ESR				CRP				
Sodium				Potassium				
Urea				Creatinine				
Bilirubin				SGPT				
SGOT				CPK				
Tourniquet test				Urinalysis (manual)				
CXR				ECG				
Other imaging results	(give details)							
Other microbiol. results [24]	(give details)							
Other investigation results	(give details)							
Discharge / Mortality Data								
Initial diagnosis [25]	UF / VF / _____			NA / probable / confirmed	DRT aware	YES / NO		
Final diagnosis [26]	UF / VF / _____			NA / probable / confirmed	DRT aware	YES / NO		
Outcome of case	discharge / left / death			Notify with final results	YES / NO			
Date of discharge / death				Cause / mode of death				
Serum samples (discharge)	YES / NO			Labelling + date of serum				
Follow-Up Data (usually 2 weeks after discharge)								
Date of follow-up				Estimated time off work				
Features at follow-up [27]	(give details)							
Knows name of disease	YES / NO			Understands transmission	YES / NO / NA			
Serum samples (follow-up)	YES / NO			Labelling + date of serum				

Appendix 1

Local Microbiology Results			
Blood films for malaria parasites	1st = NEG / POS / EQ / ND	2nd = NEG / POS / EQ / ND	3rd = NEG / POS / EQ / ND
	Species = NA / unidentified / *P. falciparum* / *P. vivax* / both species		
Panbio dengue serology (Dengue Duo Cassette)	IgM = NEG / POS / EQ / ND	IgG = NEG / POS / EQ / ND	Date :
	IgM = NEG / POS / EQ / ND	IgG = NEG / POS / EQ / ND	Date :
	IgM = NEG / POS / EQ / ND	IgG = NEG / POS / EQ / ND	Date :
MMU dengue PCR	PCR = NEG / POS / EQ / ND	Genotype =	Date :
Improved in-house blood culture	NEG / POS / CONTAMINANT / ND	Species =	
	Sensitivities =		
Manual BacT/ALERT blood culture	NEG / POS / CONTAMINANT / ND	Species =	
	Sensitivities =		
Heparinised blood culture for leptospires	NEG / POS / ND	Species =	
	Serovar =		
Urine antibiotic screen	*Strep.* = NEG / POS / ND	*E. coli* = NEG / POS / ND	*Bacillus* = NEG / POS / ND
Other microbiology tests			
Overseas Microbiology Results			
Leptospirosis ELISA (UK – LRU)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Leptospirosis MAT (UK – LRU)	Admission date :		
	Discharge date :		
	Follow-up date :		
Murine typhus serology (Thailand – Wellcome)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Murine typhus serology (UK or France)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Scrub typhus serology (Thailand – Wellcome)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Scrub typhus serology (UK or France)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Dengue serology (UK – HPA)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Chikungunya serology (UK – HPA)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Jap. encephalitis serology (UK – HPA)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Hantavirus serology (UK – HPA)	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =
Other serology	Admission date :	IgM =	IgG =
	Discharge date :	IgM =	IgG =
	Follow-up date :	IgM =	IgG =

Appendix 1

Definitions

1. "Unique identifier" = *eg.* first patient from Ragama = R001, ninety-ninth patient from Polonnaruwa = P099
2. "GN division" = Grama Niladhari (Sewaka) division = defined administrative area (similar to a postcode)
3. "Main income provider" = makes biggest contribution to family income
4. "Close contacts affected" : "neighbours" = within 100 metres, "contacts" = seen most days
5. "Water" → tick the type of water used most often for each application
6. "Washing" = non-immersion from washing pots, clothes or oneself
7. "Bathing" = immersion from showering or bathing
8. "Uncooked" = never placed in boiling water
9. "Unwashed hands" = hands were not washed with soap & water before eating
10. "Usual footwear" → circle whichever single type is worn most often during the day
11. "Skin wounds" = any break (incision, laceration or scratch) in the skin that draws blood
12. "Visited scrub jungle" = any time spent out doors in a "scrub jungle" area
13. "Definite tick bite" = tick seen at site of bite
14. "Animals" → tick the closest contact for each animal
15. "Date of onset of illness" = when symptoms first began (illness is considered "acute" if < 2 weeks duration)
16. "Fever (subjective)" = occurring before or during the first 24 hours of admission
17. "Fever (recorded)" = maximum oral temperature in first 24 hours (by glass/mercury thermometer)
18. "Fever type" : "continuous" = temperature never drops below 37.5 °C
    "intermittent" = temperature drops below 37.5 °C for < 24 hours
    "biphasic" = temperature drops below 37.5 °C for > 24 hours, but then recurs
19. "Fever duration" = from time of first temperature ≥ 38 °C until temperature < 37.5 °C for > 24 hours

    for biphasic fevers → record durations of initial fever, afebrile periods & recurrent fevers
20. "Other clinical features" : "onset" = in relation to date of arrival in hospital
    eg. feature occurring 7 days before arrival = "-7 days"
    eg. feature occurring 3 days after arrival = "+3 days"

    the duration of features that persist at discharge should be recorded as "> *x* days"
21. "↓ Glasgow Coma Scale" = lowest score as measured using the standard 15 point scale
22. "Myocarditis" = clinical diagnosis by consultant physician
23. "Investigation results" → record the first result and the "worst" (most abnormal) result during admission and also record the date of the "worst" result
24. "Other microbiol. results" = microbiology results not determined from blood samples
25. "Initial diagnosis" = most likely diagnosis at 24 hours after admission
    (UF = undifferentiated fever / VF = viral fever / DRT = dengue rapid test result)
26. "Final diagnosis" = most likely diagnosis at discharge or death
    (UF = undifferentiated fever / VF = viral fever / DRT = dengue rapid test result)
27. "Features at follow-up" = clinical features (symptoms + signs) that are either persistent or new at follow-up

Appendix 2

Consent Form for a Fever Study in Sri Lanka

Last Updated 19 November 2006

English Version

We are conducting a study in order to improve the diagnosis of infectious diseases in Sri Lanka. This work is being done in collaboration with several overseas universities, who have facilities that are not available in this country.

The study will involve recording information on patients who are admitted with a fever and performing some additional blood and urine tests (free of charge). The information recorded for the study will be stored anonymously. We estimate that the amount of blood required for the study will be 4 teaspoonfuls more than is usually required on admission and 2 teaspoonfuls more than is usually required on discharge and follow-up. Patients involved in the study will be treated in exactly the same way as other patients, but may benefit from the extra diagnostic tests being performed.

This study has been approved by the Ethics Committee of the Faculty of Medicine of the University of Kelaniya and results will be available in due course from Dr Ranjan Premaratna and Dr Shaluka Jayamanne.

I have read / heard and understood the information above.

I agree to allow information from my case to be used in this study.

I agree to have additional blood samples taken as described above.

I understand that I can choose to withdraw from this study at any time.

Name ……

Signature …….. Date ……

Sinhala and Tamil versions of this consent form are provided overleaf.

Appendix 2

ශ්‍රී ලංකාවේ පවතින ආසාදන රෝග තත්ව පිළිබඳව කරනු ලබන සමීක්ෂණය

ශ්‍රී ලංකාවේ ආසාදන රෝග නිවැරදිව හඳුනාගැනීම පිළිබඳව සමීක්ෂණයක් දැනට පැවැත්වේ. මෙම සමීක්ෂණය විදේශීය විශ්වවිද්‍යාල හා සම්බන්ධ වී කරනු ලබන අතර එමගින් අප රටේ දැනට නොමැති පරීක්ෂණ පහසුකම් ලබා ගැනීමටද අපට හැකිවී ඇත.

මේ සඳහා උණ රෝග තත්වයක් සහිතව රෝහල් ගත වන රෝගීන්ගෙන් එම රෝගය පිළිබඳව විස්තර දැන ගැනීමට අවශ්‍ය වේ. පරීක්ෂණ සඳහා අමතර මූත්‍රා හා රුධිර සාම්පල කිහිපයක් ලබා ගැනීමටද අවශ්‍ය වේ. (රෝහලට ඇතලත් වීමේදී රුධිරය තේ හැඳි 4ක් පමණ සහ රෝහලින් පිටව යාමේදී තේ හැඳි 2ක් පමණ) මෙම පරීක්ෂණය සඳහා ඔබට කිසිදු මුදලක් වැය නොවේ. ප්‍රතිකාර ක්‍රම ඵලෙසින්ම සිදු කරන අතර ඔබ මෙම පරීක්ෂණයට ඇතුලත් වීම තුලින් අප සිදුකරන අමතර විශේෂ පරීක්ෂණ මගින් ඔබගේ රෝග තත්වය ඉක්මනින් හඳුනා ගැනීමේ අවස්ථාව සැලසෙනු ඇතැයි අපි බලාපොරොත්තු වෙමු.

අප විසින් ලබා ගන්නා සියලුම තොරතුරු වල රහස්‍යභාවය සුරකින අතර ඔබට මෙම පරීක්ෂණයෙන් ඕනෑම වේලාවක ඉවත්වීමටද පුළුවන. මේ පිළිබඳව අපැහැදිලි කරුණු සහ අමතර තොරතුරු ඔබට සමීක්ෂක වෛද්‍යවරුන්ගෙන් ලබා ගත හැක.

රෝගියාගේ කැමැත්ත ප්‍රකාශ කිරීම

මා ඉහත සඳහන් තොරතුරු කියවා තේරුම් ගත් බවටත්, මෙම සමීක්ෂණය සඳහා සහභාගී වීමටත් මාගේ කැමැත්ත ප්‍රකාශ කරමි. මේ සඳහා මාගේ රෝග තත්වය පිළිබඳ තොරතුරු ලබා දීමටත්, අමතර රුධිර සාම්පල කිහිපයක් ලබා දීමටද කැමැත්ත ප්‍රකාශ කරමි.

නම : ----------

අත්සන :----------

දිනය :----------

Appendix 2

இலங்கையில் காய்ச்சல் சம்பந்தமான ஆராய்ச்சிக்கான சம்மதப் பத்திரம்

இலங்கையில் உள்ள தொற்று நோய்களை இனங்கண்டு கொள்வதற்கான ஒரு ஆராய்ச்சியினை நாங்கள் நடாத்துகின்றோம். இந்த ஆராய்ச்சியானது இலங்கையில் கிடைக்கப் பெறாத நவீன வசதிகளைக் கொண்ட பல வெளிநாட்டு பல்கலைக்கழகங்களுடன் இணைந்து நடாத்தப்படுகின்றது.

காய்ச்சல் காரணமாக வைத்தியசாலையில் தங்கியிருந்து சிகிச்சை பெறும் நோயாளிகள் சம்பந்தமான விபரங்களை தொகுப்பதும், மேலதிக தகவல்களை திரட்டும் நிமித்தம் இரத்தம் மற்றும் சிறுநீர் பரிசோதணைகளை (இலவசமாக) செய்வதும் இவ்வாராய்ச்சியில் உட்படுத்தப்படுகின்றது.

இப்பரிசோதணைகளுக்காக நோயாளிகள் வைத்தியசாலையில் அனுமதிக்கப்படுகையில் 4 தே.க. (தேநீர் கரண்டி) இரத்தமும், அந்நோயாளிகள் வைத்தியசாலையிலிருந்து வெளியேறும் போது 2 தே.க. இரத்தமும், மீண்டும் அந்நோயாளிகள் மீள்பரிசோதனைக்கு வரும் போது 2 தே.க. இரத்தமும் எடுக்கப்படும் என நாம் அனுமானிக்கின்றோம்.

இவ்வாராய்ச்சியில் உட்படுத்தப்படும் நோயாளிகள் ஏனைய சாதாரண நோயாளிகளுக்கு வழங்கப்படும் சிகச்சை முறையையே பெறுவர். ஆயினும் இவ்வாராய்ச்சி பரிசோதனை மூலம் நோயாளிகள் மேலதிக பரிசோதனைகள் செய்வதற்கான பயனை பெறலாம்.

இவ்வாராய்ச்சியானது களனி பல்கலைக்கழக வைத்திய பீடத்தின் அனுமதியின் கீழ் நடாத்தப்படுவதுடன் இப்பரிசோதனை முடிவுகள் உரிய நேரத்தில் வைத்தியர்கள் Dr.இரஞ்சன் பிரேமரத்னவிடமும், Dr.ஷாலுகா ஜயமான்னவிடமும் வழங்கப்படும்.

- நான் மேற்குறிப்பிட்ட விபரங்களை வாசித்து/கேட்டு விளங்கிக் கொண்டேன்.
- எனது நோய் சம்பந்தமான விபரங்களை இவ்வாராய்ச்சிக்கு உட்படுத்த சம்மதிக்கின்றேன்.
- பரிசோதணைகளுக்காக தேவைப்படும் இரத்தத்தை வழங்க சம்மதிக்கின்றேன்.
- இவ்வாராய்ச்சியிலிருந்து எந்த நேரத்திலும் என்னால் விலகிக் கொள்ள முடியும் என்பதையும் அறிந்துள்ளேன்.

பெயர் _____

கையொப்பம் _____ திகதி _____